An ECM-based model derived from DWJM to study hematopoiesis and leukemia

By

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Abstract

Hematopoietic stem cells (HSCs) are responsible for the generation of the body's whole blood and immune cells. This process occurs mainly within the bone marrow microenvironment, known as the "niche", which provides signals to regulate HSC survival, quiescence, self-renewal, and differentiation. This niche microenvironment is comprised of cellular components, extracellular matrix (ECM), and soluble cytokines and growth factors. Although the understanding of these signals is growing, there is still no well-established *in vitro* system to study the role of these signals in hematopoiesis; such knowledge could be utilized to expand HSCs for clinical use. In addition, a growing number of studies have shown that alteration of the bone marrow niche is associated with leukemia resistance and progression. Here we used decellularized Wharton's jelly matrix (DWJM), the ECM part of umbilical cord, as a scaffold to engineer a hematopoietic niche for normal and malignant hematopoiesis. The findings that DWJM prevents the loss of hematopoietic stem cell characteristics and that it enriches a leukemia stem cell (LSC) – like population indicate that this natural ECM biomaterial is a suitable *in vitro* model of the bone marrow microenvironment that could be utilized to expand HSPCs (hematopoietic stem and progenitor cells) and to provide a potential platform to study the effects of niche components on hematopoiesis and leukemia.

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Chapter 1:

General Introduction

Hematopoiesis and hematopoietic stem cells (HSCs)

Hematopoiesis is a process in which the pluripotent HSCs continuously and dynamically give rise to various types of blood cells. During development, primitive hematopoiesis occurs in the yolk sac, and followed by definitive hematopoiesis in the aorta-gonad-mesonephros (AGM) region, then in the fetal liver, spleen, and finally in bone marrow (Wang and Wagers 2011). Except for some specialized lymphocytes, mature blood cells are short-lived and thus need to be replenished throughout life to maintain homeostasis. HSCs, which are the cells at the top of the hematopoietic hierarchy, are responsible for hematopoietic homeostasis. After each cell division, one HSC can generate at least one daughter cell exactly the same as the parent, known as self-renewal, and another cell which can subsequently differentiate into all the hematopoietic lineages, referred to as multipotency (Bylinkina, Golubeva et al. 1991, Weissman, Anderson et al. 2001). In the bone marrow, the highly dormant and long-term repopulating HSCs (LT-HSCs) are a reserve pool of the most potent HSCs, and are activated only during stress or injury; once active, the self-renewing short-term HSCs (ST-HSCs) are mainly responsible for hematopoietic homeostasis (Ehninger and Trumpp 2011). Cell differentiation occurs from the stem cells to multipotent progenitors (MPPs), and then to myeloid or lymphoid progenitors, which have restricted developmental potentials, and subsequently generate all the myeloid (erythrocytes, platelets, granulocytes, macrophages) and lymphoid cells (T cells, B cells, nature killer cells). HSCs can also migrate to the circulation and home back to bone marrow

especially via signaling molecules like granulocyte colony-stimulating factor (G-CSF), or in response to chemotherapy, or under stress. The circulating HSCs also contribute to hematopoiesis, mainly by entering the peripheral hematopoietic organs like spleen and liver, and by triggering increased hematopoietic cell proliferation and differentiation under stress (Trumpp, Essers et al. 2010).

One barrier for studying HSCs is the identification of this rare population (Kiel, Yilmaz et al. 2005). Perhaps the best way to identify HSCs is to use serial transplantation to test stemness (Lemischka, Raulet et al. 1986). NSG (NOD scid gamma) mice is currently the most widely used immunocompromised model and serve as a useful tool for *in vivo* analysis of human HSCs(Shultz, Ishikawa et al. 2007). However, there is no ideal *in vitro* assay to specifically identify these cells. Colony-forming assay detects cells that give rise to large colonies but they can also be short-term progenitors. Long-term culture initiating cell (LTC-IC) assays can detect some but not all HSCs, which are more primitive than colony forming cells.

Since overwhelming studies of hematopoiesis are based on mouse models, the isolation and identification of mouse HSCs are more well-established. HSCs and MPPs do not express the surface markers that are associated with the terminal differentiation of specific blood cell types. Lack of expression of these lineage (lin) markers including CD3 (T cell), B220 (B cell), Gr1 (granulocyte), Mac1 (monocyte), and Ter119 (erythrocyte) which includes all of the mature hematopoietic cells can be used to isolate mouse hematopoietic stem and progenitor cells (HSPCs) from the more differentiated cells.

Further, murine bone marrow HSCs do not express cell-surface markers of lineage-committed hematopoietic cells (Lin^-) but do express high levels of Sca-1 and c-Kit, referred to as the LSK ($Lin^-Sca\text{-}1^+c\text{-}Kit^+$) cells. Additional markers like CD150, CD244, and CD48 increased the specificity (Kiel, Yilmaz et al. 2005). Other studies have indicated that LSK in combination with Flk2 and CD34 can be used to identify long-term reconstituting HSCs (LT-HSCs, $CD34^-FLT3^-$) (Osawa, Hanada et al. 1996), short-term HSCs (ST-HSCs, $CD34^+FLT3^-$) and multipotent progenitors (MPP, $CD34^+FLT3^+$) (Purton and Scadden 2007).

Like in mouse, primitive human hematopoietic cells do not express lineage specific markers (i.e., CD3, CD4, CD8, CD14, CD15, CD16, CD19, CD20, CD56, and CD66b). In human, CD34 is the most widely accepted marker for the primitive hematopoietic cells to enrich HSPCs for both research and clinical use. The frequency of $CD34^+$ cells is 1-4% of the nucleated cells in normal human bone marrow (BM) aspirate and <0.1% of nucleated cells in human peripheral blood under homeostasis (Eaves, Glimm et al. 2001). However, many of $CD34^+$ cells are lineage-restricted progenitors while HSCs is still a small proportion within $CD34^+$ cells. Efforts to purify HSCs have been done based on efflux of vital dyes like Hoechst, functional markers such as the detoxifying enzyme aldehyde dehydrogenase (ALDH) (Frandberg, Borestrom et al. 2015). Other studies have shown that HSCs can be enriched further on the basis of CD45RA, Thy1,and CD38 expression (Baum, Weissman et al. 1992, Craig, Kay et al. 1993, Mayani, Dragowska et al. 1993, Hao, Shah et al. 1995, Bhatia, Wang et al. 1997, Majeti, Park et al. 2007). Then CD49f was used to distinguish HSCs from MPP, and this is so far the most convincing

combination of cell surface markers to label LT-HSCs, ST-HSCs and MPPs tested based on transplantation in mouse model (Notta, Doulatov et al. 2011).

Since HSCs are responsible for producing the whole hematopoietic system, mutations occurring during hematopoiesis can lead to many diseases like leukemia, myeloma, lymphoma, and anemia. Since many of those diseases are fatal even with drug treatment, HSC transplantation (HSCT) is widely used to treat patients with life-threatening hematologic diseases. HSCT involves the elimination of a patient's blood and immune system by chemotherapy or radiotherapy, and replacing it with hematopoietic stem and progenitor cells (HSPC) either from another donor or the patient's own previously collected stem cells. Traditionally, stem cells are collected from bone marrow and peripheral blood. To collect stem cell from bone marrow, the clinical practitioners give the donor a general anesthetic and 500-1200ml bone marrow aspirate is harvested from the pelvis. The mononuclear cells are counted to estimate the yield. This collection process is invasive, and donors undergo severe pain without anesthesia. Peripheral blood stem cells are collected by a cell separator instrument connected to the donor via peripheral cannula. The donor's blood goes through the cannula and pumped into the cell separator instrument. The mononuclear cells are collected by the machine and the red blood cells go back to the donor's blood circulation. This process, called apheresis, takes a few hours to get enough cells for transplantation. Because during homeostasis, the number of hematopoietic stem cells in the blood circulation is extremely low, chemotherapy (only in autologous transplantation) and growth factors

(usually G-CSF) are administrated into the donors to increase the stem cells before collection of peripheral stem cells. This collection method is dependent on the stem cell mobilization, so sometimes it requires repeated collections. A cell dose of 2-4x10^8 nucleated cells /kg recipient body weight is needed, if the stem cell source is bone marrow, or 2.5x10^6 $CD34^+$ cells/kg body weight, if the source is peripheral blood, for successful transplantation. Cord blood is another source of stem cells used in transplantation, especially in pediatric patients, since fetal blood contains a large number of hematopoietic stem cells. An estimated 20,000 umbilical cord blood transplants have been performed worldwide to treat patients with malignant and nonmalignant diseases (Aljitawi 2012, Lund, Boitano et al. 2015). Compared with bone marrow and peripheral blood, cord blood not only contains higher frequency of HSPCs, but is also associated with lower rate of graft-versus-host disease (GVHD) post-transplantation. The source of cord blood normally is limited and the amount is small with each transplantable unit often ranging in volume from 60 to 100ml. On the other hand, the hematopoietic reconstitution ability of the stem cells in cord blood is higher than other stem cell sources, thus a lower cell dose of CD34+ cells/kg body weight is needed compared with peripheral blood (Ballen, Gluckman et al. 2013).

Hematopoietic stem cell niche

Under normal conditions, HSCs reside in a specialized bone marrow microenvironment, also known as the stem cell niche. The bone marrow is a soft structure within the highly vascularized trabecular bone and is the main site for hematopoiesis (Gurkan and Akkus

2008). Since the term “stem cell niche” was first proposed by Schofield more than 30 years ago (Schofield 1978), many studies have been focused on illustrating the components and their role in HSC self-renewal and differentiation. Niche components regulate the fate of HSCs by direct cell–cell contact, or by producing a range of growth factors, cytokines, chemokine, and extracellular matrix (ECM) (Lymperi, Ferraro et al. 2010). The stem cell niche is a highly dynamic microenvironment which does not only influence the HSCs and the hematopoietic cells derived from HSCs, but could also be remodeled by them.

1. Cellular components of HSC niche

Endothelial cells. So far, multiple niche components have been elucidated for HSCs. Recent studies based on advanced bone marrow imaging technologies and genetic ablation mouse models have indicated that the HSCs are mostly perivascular (Xie, Yin et al. 2009, Kunisaki, Bruns et al. 2013, Nombela-Arrieta, Pivarnik et al. 2013, Acar, Kocherlakota et al. 2015). In the perivascular niche, cells of the vasculature are of great interest. Actually, it has been shown that the endothelial cells and hematopoietic cells are from the same precursor—the hemangioblast (Shalaby, Rossant et al. 1995, Kennedy, Firpo et al. 1997). These cells are usually identified by their expression of the endothelial-specific markers CD31, MECA-32, V-cadherin and vascular endothelial growth factor receptor 2 (VEGFR2), and line the surface of blood vessels, connecting the blood and tissues in the bone marrow. Imaging analyses reveal that HSPCs can be found co-localized with the marrow vasculature both in the central bone and endosteal

regions (Xie, Yin et al. 2009, Kunisaki, Bruns et al. 2013, Nombela-Arrieta, Pivarnik et al. 2013). This supports the concept that endosteal and vascular niches are not absolutely independent from each other but exists in an overlapping manner; since there is a dense vascularization within the bone marrow. *In vivo* studies suggest that vascular endothelial cells are critical for maintenance of HSPCs within bone marrow as well as their function of hematopoietic system reconstitution after bone marrow injury (Li, Bailey et al. 2010, Ding, Saunders et al. 2012). Endothelial cells regulate the HSC retention in bone marrow, self-renewal and differentiation by expressing many factors in both contact dependent and independent ways. These factors include, but not limited to, secreted proteins like stem cell factor (SCF) and stromal cell-derived factor 1 (SDF1) (Kennedy, Firpo et al. 1997, Ding, Saunders et al. 2012, Ding and Morrison 2013, Greenbaum, Hsu et al. 2013), Notch ligands(Butler, Nolan et al. 2010), angiopoietin 1 (ANGPT1)(Arai, Hirao et al. 2004), pleiotrophin (Himburg, Harris et al. 2012), and epidermal growth factor (EGF) (Doan, Himburg et al. 2013), and membrane-bound proteins such as E-selectin, which promotes HSC proliferation and increases HSC chemo-sensitivity (Winkler, Barbier et al. 2012). Bone marrow-derived endothelial cells have shown some capacity to facilitate HSC ex vivo expansion (Rafii, Shapiro et al. 1995, Butler, Nolan et al. 2010), while endothelial cells from other sources showed varying degrees of expansion support (Avecilla, Hattori et al. 2004, Qu, Liu et al. 2016). Limited *in vivo* data suggests the vascular niche may be particularly important in the activation of HSCs for hematopoiesis, as shown by enhanced proliferating HSCs recruitment to the perivascular region observed following bone marrow stress (Heissig, Hattori et al. 2002, Avecilla, Hattori et al.

2004).

Perivascular MSCs. Among all of the cells in the bone marrow microenvironment, mesenchymal stem cells have long been studied for their roles in HSC fate decision. As a rare cell population within the bone marrow (1 in 10,000 nucleated cells) (Friedenstein, Chailakhjan et al. 1970), they are able to give rise to the entire mesenchymal lineages including bone, cartilage and fat cells, as well as various fibroblast-like stromal cells producing HSC supporting factors and serving as scaffolds alone or by producing ECM components in the bone marrow (Pittenger, Mackay et al. 1999, Liechty, MacKenzie et al. 2000, Lin, Moreno-Luna et al. 2014). The unique properties of this cell population make them popular in the research of regenerative medicine and tissue engineering. Previous studies have focused on elucidating the specific cell surface marker for the proper isolation and identification of MSCs but the conclusions are still inconsistent. Diverse antigens have been found on the surface of MSC, but none of them is unique to the cells (Rostovskaya and Anastassiadis 2012, Lv, Tuan et al. 2014). According to the International Society for Cellular Therapy (ISCT), the minimal criteria for MSCs are: (1) adhesion to plastic culture dish under the standard culture conditions; (2) expression of CD105, CD73 and CD90, but not lineage markers such as CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR; (3) the ability to differentiate into osteoblasts, adipocytes and chondrocytes *in vitro* (Dominici, Le Blanc et al. 2006). Some recent studies based on transgenic mouse models found that the bone marrow MSCs located adjacent to vasculature are important for the HSC maintenance and cell fate decision

((Mendez-Ferrer, Michurina et al. 2010; Ding, Saunders et al. 2012; Kunisaki, Bruns et al. 2013). Different markers have been used to identify these cells including nestin (Nes)-GFP (Mendez-Ferrer, Michurina et al. 2010), leptin receptor (LEP-R) (Ding, Saunders et al. 2012), neuron/glial antigen 2 (NG2) (Kunisaki, Bruns et al. 2013), Paired related homebox 1 (Prx-1) (Greenbaum, Hsu et al. 2013), platelet-derived growth factor receptor alpha (PDGFRA or CD140a), CD51 and Sca-1 (Pinho, Lacombe et al. 2013), CD146 (Sacchetti, Funari et al. 2007) and CD271 (Matsuoka, Nakatsuka et al. 2015). Although the markers used in these studies are different from each other, they have all been shown to support HSC self-renewal and multipotency. However, the distribution of HSCs and the MSC population having direct contact with them remain controversial. While NG2+LEP-R^{-} peri-arteriolar MSCs close to endosteal area were first reported to be associated with quiescent HSCs, and NG2−LEP-R^{+} peri-sinusoidal MSCs which are peri-sinusoidal and located in central bone away from the endosteum are associated with activated HSCs (Kunisaki, Bruns et al. 2013), a recent study indicated that both the quiescent and active HSCs prefer to localize in the peri-sinusoidal area with LEP-R+ MSCs (Acar, Kocherlakota et al. 2015). However, these findings from both these groups illustrate the importance of CXCL12 and SCF expressed by the MSCs. Actually, the perivascular stromal cells expressing high amounts of CXCL12, known as CXCL12-abundant reticular (CAR) cells, maintain the quiescent HSC pool by regulating their self-renewal, proliferation and trafficking (Sugiyama, Kohara et al. 2006). It seems that these cells are more differentiated progenitors with adipogenic and osteogenic potential (Omatsu, Sugiyama et al. 2010). So far, it is widely recognized that these

perivascular MSCs play an important role in the maintenance of HSCs within the bone marrow niche shown by many *in vivo* studies based on genetically engineered mice. These studies either ablated the whole MSC population expressing CXCL12 (Omatsu, Sugiyama et al. 2010), or specifically knocked out CXCL12 or SCF in Lep-R^+ MSCs (Ding, Saunders et al. 2012, Ding and Morrison 2013).

Megakaryocytes. Megakaryocytes are terminally differentiated hematopoietic cells responsible for the production of platelets. The concept that megakaryocyte population is an essential part of HSC niche was just established in recent years (Bruns, Lucas et al. 2014, Nakamura-Ishizu, Takubo et al. 2014, Zhao, Perry et al. 2014). Previous studies found that after transplantation HSCs lodge near megakaryocytes (Heazlewood, Neaves et al. 2013), and the indirect influence of megakaryocytes on HSCs is due to its promotion of osteoblastic niche expansion (Olson, Caselli et al. 2013). Then recent studies discovered that megakaryocytes also directly regulate HSCs as they promote HSC quiescence by secreting CXCL4 (Bruns, Lucas et al. 2014), TGF-β1 (Zhao, Perry et al. 2014) and TPO (Nakamura-Ishizu, Takubo et al. 2014) during homeostasis, and activate HSCs by producing FGF1 under regenerative stress (Zhao, Perry et al. 2014). However, whether the activation is at the expense of losing self-renewal ability is still not clear. The location of these megakaryocytes is peri-sinusoidal, indicating that these cells are part of the sinusoid niche independent of arterioles. Since a recent study also found that most quiescent HSCs reside in peri-sinusoidal niche, whether the endothelial cells, pericytes

and megakaryocytes cooperate or each has a distinct role in regulating HSC quiescence is worth studying in the future.

Osteoblasts. The role of the bone-forming osteoblasts is controversial. The endosteal niche was originally proposed since the first *in vivo* studies demonstrated that osteolineage cells, the interface between the bone and the bone marrow had direct contact with HSCs and were required for HSC maintenance by BMP signaling and Notch signaling (Calvi, Adams et al. 2003, Zhang, Niu et al. 2003). Differentiating osteoblasts express many factors important for HSC function including CXCL12, SCF and ANGPT1 (Blair, Dong et al. 1999, Shahnazari, Chu et al. 2013, Zhou, Ding et al. 2015). They also produce extracellular matrix proteins including collagen, fibronectin, laminin, vitronectin, osteopontin and osteonectin (El-Amin, Lu et al. 2003). However, some later studies have shown that some osteolineage cells are partially dispensable in adult BM without compromising the long-term HSC maintenance under homeostasis (Ding, Saunders et al. 2012, Ding and Morrison 2013, Frenette, Pinho et al. 2013, Greenbaum, Hsu et al. 2013). According to these studies, deletion of *Scf* or *Cxcl12* expression in osteoblasts does not affect HSC number and hematopoietic reconstitution ability but rather reduces the numbers of B lymphoid progenitors (Ding and Morrison 2013, Greenbaum, Hsu et al. 2013). *In vitro* B cells differentiation requires attachment to osteoblasts through the effects of VCAM-1, SDF-1, and IL-7 signaling induced by parathyroid hormone (PTH) (Rankin, Wu et al. 2012). Osteoblasts also produce EPO to modulate erythropoiesis through HIF signaling (Rankin, Wu et al. 2012). All together it seems that these

bone-forming cells play a more important role in the lineage commitment than the maintenance of HSCs.

Other cellular components in bone marrow niche. Some other cell types have also been shown to regulate HSC quiescence and activation, self-renewal and lineage commitment. These include macrophages, sympathetic nerve system, and adipocytes (Katayama, Battista et al. 2006, Naveiras, Nardi et al. 2009, Mendez-Ferrer, Michurina et al. 2010, Winkler, Sims et al. 2010, Chow, Lucas et al. 2011). Macrophages regulate HSC trafficking and differentiation by interacting with MSCs and osteoblasts to regulate their expression of SCF, CXCL12, and IL7 (Winkler, Sims et al. 2010, Chow, Lucas et al. 2011). The noradrenergic nerves functions to regulate HSC trafficking by interacting with nestin+MSCs through their β3-adrenergic receptor, resulting in the down regulation of HSC maintenance genes SCF, CXCL12, Angpt1 and vascular cell adhesion molecule 1 (Vcam-1), making HSCs egress from the bone marrow (Katayama, Battista et al. 2006, Mendez-Ferrer, Michurina et al. 2010). Nonmyelinating Schwann cells wrapping around sympathetic nerves, help keep HSCs quiescent by expressing TGF-β1 (Yamazaki, Ema et al. 2011). Adipocytes are considered as a negative regulator in the bone marrow niche since HSC engraftment is accelerated after bone marrow adipocyte ablation (Naveiras, Nardi et al. 2009).

2. *Extracellular matrix.*

In the BM, a complex network of extracellular matrix (ECM) including but not limited to fibronectins, laminins, collagens, and proteoglycans support hematopoiesis. By imaging

the main ECM proteins in mouse bone marrow, researchers have found that these proteins have different distributions in the bone marrow, suggesting that they play different roles in HSC homing and lodgment after transplantation (Nilsson, Debatis et al. 1998). The ECM affect the HSCs in both direct and indirect ways, since it can bind to cell surface receptors directly, or serve as a reservoir for soluble growth factor that can be presented to HSCs. Integrins, the heterodimeric transmembrane molecules consisting of an α and β subunits (Hynes 1992), are the most widely studied cell-surface receptors binding to ECM thus inducing homing, adhesion, survival, proliferation and differentiation of HSCs in the niche (Legate, Wickstrom et al. 2009). For example, tenascin-C produced by stromal cells and endothelial cells, is up-regulated during hematopoietic recovery after myeloablation. Although knocking out tenascin showed normal steady-state hematopoiesis in mice, it could be fatal after bone marrow ablation; *in vitro* culture of HSCs on tenascin-C promoted their proliferation via its receptor integrin α9, suggesting this ECM protein is a critical component of the bone marrow microenvironment required for hematopoietic reconstitution (Nakamura-Ishizu, Okuno et al. 2012). Integrin α6, the receptor of laminin, collaborates with integrin α4 to facilitate the homing of HSCs after transplantation (Qian, Tryggvason et al. 2006). Deletion of integrin α4 results in the HSCs leaving bone marrow to the blood circulation and accumulation in the spleen under homeostasis, delayed erythroid and myeloid progenitor regeneration under stress, and impaired homing and engraftment after transplantation (Scott, Priestley et al. 2003). Mouse HSCs cultured in fibronectin and laminin expanded CFU-GEMM while increasing the cell surface proteins CD29, VLA-4, and MDR, which are all the characteristics of

primitive HSCs (Sagar, Rentala et al. 2006). A few non-integrin receptors also have been found to be important in HSC trafficking and lodgement. For example, CD44, the receptor of glycosaminoglycan hyaluronan, cooperate with SDF-1 to mediate HSC homing after transplantation (Sagar, Rentala et al. 2006).

Engineering HSC niche *in vitro*

The clinical use of HSCs in transplantation to treat patients suffering from hematological disorders relies on their potential to reconstitute all the hematopoietic system. However this usage, especially for cord blood HSC transplantation is hampered by the limited availability because of the low cell number collected each time regardless of the source. Currently the most elegant solution for overcoming the problem is to expand these cells *ex vivo* prior to infusion. The manipulation of these cells *in vitro* will not only allow the enhanced availability of a larger cell population for HSCs, but also can potentially increase their homing ability if using appropriate methods to induce the “homing factors” such as CXCR4 and C3a receptor (Lund, Boitano et al. 2015), to improve clinical outcomes. *In vitro* mimicking the niche where hematopoiesis occurs would help understand the interactions between HSCs and their microenvironment including the niche cell, and biophysical / bimolecular signals, thus leading to successful expansion of HSCs while maintaining their stem cell properties. To date, a variety of culture systems have been established to elucidate the microenvironmental cues on HSC fate decisions. The design of these systems mainly include biomaterial-based culture platforms,

co-culture with different niche cells, and a three dimensional (3D) biomaterial-based co-culture system.

Two dimensional (2D) culture systems. In vitro culture of hematopoietic cells can be stroma-free or stroma-dependent. Stroma-free cultures are the most conventional cell suspension in medium supplemented with cytokine cocktails. It is easy to perform and control specific factors influencing HSC fate decision. However, given that more and more evidence has shown niche cells have significant effects on the survival, proliferation, quiescence, self-renewal and differentiation of HSCs, co-culture of HSCs with these feeder cells is a common strategy to study the cell-cell interaction in bone marrow niche. Bone marrow stromal cells are the most commonly used cell population for ex vivo expansion of HSCs (Verfaillie 2002, Sauvageau, Iscove et al. 2004). Here, culture with bone marrow MSCs (Jing, Fonseca et al. 2010), endothelial cells (Chute, Saini et al. 2002), and osteoblasts (Chute, Saini et al. 2002) have all been shown to expand HSCs while maintain their long-term reconstitution ability. Feeder cells can also induce the differentiation of HSPCs into committed lineages including erythroids, megakaryocytes, NK cells, T cells, and B cells (Vieira and Cumano 2004, Giarratana, Kobari et al. 2005, Taqvi, Dixit et al. 2006, Dezell, Ahn et al. 2012). The feeder layers functions both by direct cell-cell contact via adhesion molecules, or by secreting cytokines and growth factors, acting in a paracrine manner.

Scaffold-based three dimensional (3D) cell cultures. The natural bone marrow niche is a 3D soft marrow where HSCs reside in the highly viscous and porous spongiosa

environment. This 3D environment consists of structural elements and signals that affect the cells from all aspects, including the local accumulation and depletion of diffusible growth factors. Such effects cannot be recapitulated by the conventional 2D culture systems. Highly porous scaffolds have been used as culture platforms for HSCs. The 3D porous materials provide more space than the 2D conditions thus facilitate higher cell densities (Li, Ma et al. 2001). In addition, such scaffolds help construct the biophysical and mechanical function of the bone marrow microenvironment. Mouse ESCs tended to differentiate into HSCs in PLLA scaffolds with smaller pore sizes and higher polymer concentrations, indicating that pore size and viscosity are important in HSC fate decision (Taqvi and Roy 2006). Another study compared several commonly used scaffolds (PCL, PLGA, fibrin, collagen) to culture cord blood HSCs, showing that fibrin pre-seeded with MSCs support HSC expansion mostly, while 3D PLGA scaffolds led to poor HSC survival and did not induce adhesion of HSCs, suggesting that not only the pore size, but the composition of scaffolds is also important; scaffolds with natural BM ECM components support HSC growth better than synthetic ones (Taqvi and Roy 2006). Culture wells coated with collagen preseeded with MSCs promoted HSPC proliferation while forming cobblestone-like clusters, as well as the formation of a sinusoid-like structure indicate that by using an ideal ECM mimicking material, it is highly possible to recapitulate the bone marrow niche *in vitro* (Leisten, Kramann et al. 2012).

Although the scaffold-based 3D culture systems facilitate the distribution of stromal and hematopoietic cells thus increase cell-matrix interactions and mimic the stem cell niche

for expansion of the desired cell type for potential clinical use, there are still some unknowns in terms of the design and fabrication of such 3D scaffolds. Although there is growing knowledge on HSCs in cell and molecular biology perspective, the impact of bone marrow microenvironment biophysical properties on HSC fate decision still requires exploration. The design, reproducibility and standardization of the scaffold structure, and the subsequent standardized culture protocols are areas need to be addressed and optimized for clinical usage.

The bone marrow niche in acute myeloid leukemia (AML)

In the bone marrow, the hematopoietic stem and progenitor cells are exposed to various stresses which would cause mutations, and the accumulation of the mutations can then give rise to leukemia stem cells (LSCs). The mutations can occur both in the highly self-renewal HSCs stage and more committed myeloid progenitors, since LSCs have been found with phenotypes of both (Goardon, Marchi et al. 2011). These characteristics of LSCs make it difficult to find out specific markers to isolate them. So far one of the best ways to characterize LSCs is to test their ability to initiate this disease in serial transplantations based on animal models. Since people believe that LSCs reside within bone marrow and can cause relapse even after the chemotherapeutic ablation of all the leukemia cells in the circulation, as well as the fact that it is difficult to culture patient leukemia samples *in vitro*, it is highly possible that LSCs depend on the bone marrow niche for their survival and propagation like HSCs. In addition, as the propagation of malignant hematopoietic cells is at the expense of normal hematopoiesis, LSCs may

exploit the HSC niche, and further modulate the niche components on which normal cells depend (Schepers, Campbell et al. 2015). Examining the interaction between leukemia and hematopoietic niche may provide opportunities for treatment design.

Like solid tumors, highly proliferating AML cells require increased angiogenesis in many patients (Hussong, Rodgers et al. 2000, Padro, Ruiz et al. 2000). The expansion of vasculature in the bone marrow of AML patients is usually associated with poor prognosis (Rabitsch, Sperr et al. 2004). Studies have found that AML cells localize to endosteal niche (Hatfield, Oyan et al. 2009), inducing proliferation of microvascular endothelial cells through secreting CXCL8 (Cogle, Goldman et al. 2014). On the other hand, in the vascular niche a subset of AML cells adhere to endothelial cells through the cell adhesion molecule E-selectin or even fuse with endothelial cells to enter a quiescence state which give rise to chemoresistance (Pezeshkian, Donnelly et al. 2013, Cogle, Goldman et al. 2014). Interestingly these leukemia-derived endothelial cells are able to initiate leukemia after transplantation suggesting that these cells maintain a LSC function (Cogle, Goldman et al. 2014). In addition, treating endothelial cells with vascular disrupting agents could increase AML cell apoptosis through the production of reactive oxygen species, indicating that targeting endothelial cells might be a good strategy for treating AML (Madlambayan, Meacham et al. 2010).

Similarly, there is also remodeling of endosteal niche in leukemic bone marrow. In mouse models, LSCs have been shown to localize next to osteoblasts (Ninomiya, Abe et al. 2007), and osteoblasts protect AML cells from SDF1a-induced apoptosis (Kremer,

Dudakovic et al. 2014). Also it appears that malignant myeloid cells can stimulate mesenchymal stromal cells (MSCs) to produce osteoblasts through up-regulation of RUNX2 and this induces chemo-resistance (Schepers, Pietras et al. 2013). The activation of MSC to osteoblastic cell-specific differentiation by parathyroid hormone (PTH) receptor enhances AML after transplantation into mouse models via increased TGF-β1 signaling on the LSCs (Krause, Fulzele et al. 2013).

The functions of bone marrow stromal cells (MSCs) on AML have been partially elucidated so far. MSCs can provide protective signals supporting survival and chemoresistance of AML cells in animal models through SDF-1/CXCR4 axis, VCAM-1/VLA-4 interaction, and induction of quiescence (Matsunaga, Takemoto et al. 2003, Zeng, Shi et al. 2009, Jacamo, Chen et al. 2014). Studies have also indicated that AML cells produce IL10 and G-CSF to enhance the production of growth arrest-specific gene 6 (Gas6) by MSCs, thus promotes AML cell survival and chemoresistance. In fact, clinical data have reported that GAS6 expression identified high-risk adult AML patients by shorter disease-free and overall survival rate, probably by overexpression of BAALC and MN1, SDF-1 and its receptor CXCR4/CXCR7 (Whitman, Kohlschmidt et al. 2014). AML cells could also disrupt normal hematopoiesis while increasing BM infiltration of AML cells by inducing loss of HSC supporting peri-arteriolar NG2+ MSCs and nerve fibers, resulting in the down-regulation of SDF-1 and SCF which are necessary for the maintenance of HSCs in bone marrow (Hanoun, Zhang et al. 2014).

Altogether, these studies demonstrate that AML cells would not only recognize and hijack the bone marrow niche for normal HSCs, but can also transform the niche to a leukemia-favorable microenvironment to support the survival of malignant cells. Since patient leukemia cells are difficult to maintain viability *in vitro*, a cell culture model mimicking the bone marrow niche to support the long-term culture of patient samples is needed to study the biology of leukemia-niche interactions as well as drug development.

Chapter 2

Characterization of a novel Wharton's jelly ECM based model mimicking the hematopoietic niche

Abstract

HSCs reside in the “BM hematopoietic niche”, a special three-dimensional (3D) microenvironment that regulates the HSCs self-renewal and multi-potency. In this study, we established a 3D culture system that recapitulates the BM hematopoietic niche to expand hematopoietic stem and progenitor cells (HSPCs). We used decellularized Wharton’s jelly matrix (DWJM) as extracellular matrix (ECM) scaffold and human bone marrow (BM) mesenchymal stromal cells (MSCs) as supporting niche cells. . We assessed the efficacy of the DWJM-based 3D culture condition with or without BM MSCs by analyzing proliferation, apoptosis, self-renewal, lineage differentiation, and homing potential of umbilical cord CD34+ (UCBCD34+) HSPCs. Our results indicate that DWJM alone promotes HSPC quiescence while maintaining their viability and clonogenic capacity. DWJM culture also increases frequency of c-kit+ HSPCs, a population with enhanced self-renewal ability. Furthermore, DWJM alone induces expression of CXCR4, thereby enhancing HSPC migration toward stromal cell-derived factor-1 (SDF-1) .
In contrast, DWJM with BM MSCs highly stimulates proliferation of HSPCs and decreases their transmigration potential towards SDF-1 by suppressing CXCR4 expression. In conclusion, the DWJM and BM MSC culture condition provides a model system to study importance of distinct components of BM hematopoietic niche on HSPC quiescence, proliferation and differentiation and could potentially serve as a HSPC expansion system for clinical use.

Introduction

Hematopoiesis is the process of generating blood and immune cells in the body from a very small number of hematopoietic stem cells (HSCs). HSCs reside in the “BM hematopoietic niche”, a special microenvironment that regulates the HSCs self-renewal and multi-potency. Since the “niche” concept was first proposed by Schofield in 1978 (Schofield 1978), many studies have focused on understanding the niche-HSC interactions by using both *in vivo* mouse models and *in vitro* culture systems (Butler, Nolan et al. 2010, Ding, Saunders et al. 2012, Ding and Morrison 2013, Li, Ghazanfari et al. 2014, Acar, Kocherlakota et al. 2015, Zhou, Ding et al. 2015, O'Hagan-Wong, Nadeau et al. 2016). The main components of bone marrow (BM) hematopoietic niche are the HSC surrounding cells including MSCs, osteoblasts and endothelial cells. In addition, multiple ECM proteins (collagen, fibronectin, tenacin) along with cytokines and growth factors, which bind or diffuse into ECM are important components of the BM niche (Schepers, Campbell et al. 2015).

The BM components control the size of the HSC pool and regulate the HSC fate during normal homeostasis and under stress. For example, MSCs, which are the precursor cells of all mesenchymal cell types in the hematopoietic niche like adipocytes, osteoblasts and fibroblasts, play a critical role in HSC self-renewal and mobilization (Ding, Saunders et al. 2012, Ding and Morrison 2013, Acar, Kocherlakota et al. 2015, Zhou, Ding et al. 2015). ECM components also influence the trafficking and lodging of HSCs by interacting with their receptors on the cells surface. It has been postulated that

the calcium sensing receptor on HSCs could increase CXCR4-SDF-1 signaling and promote HSCs adherence to collagen in the ECM (Lam, Cunningham et al. 2011). Also hyaluronic acid in ECM could synergize with SDF-1 to enhance the homing ability of HSCs into BM (Avigdor, Goichberg et al. 2004). However, the precise mechanisms by which the BM niche components regulate HSC quiescence vs. proliferation vs. differentiation is still poorly understood.

The nature of HSCs makes them widely used in stem cell transplantation to treat malignant and non-malignant hematopoietic diseases as well as metabolic disorders. The main sources of HSCs are BM, peripheral blood, and cord blood. Compared to peripheral blood and BM, cord blood HSCs are non-invasively collected, have a greater proliferation potential, require less HLA matching, and when transplanted are associated with decreased rates of transmissible infections and graft-versus-host disease (GVHD) (Aljitawi 2012). However, cord blood HSC transplantation is usually associated with higher rates of graft failure, delayed engraftment, and poor immune reconstitution (Aljitawi 2012), mainly because of the lower cell dose, and probably the relatively high proportion of more primitive $CD34^{+}CD38^{-}$ stem cells but much lower proportion of committed progenitors (Gomi, Hasegawa et al. 1997, Wang, Doedens et al. 1997), which contribute to short-term hematopoietic reconstitution. Currently the impact of different components of BM niche on niche-HSC interactions are mostly studied *in vivo* mouse models, mainly through knocking out some specific molecules produced by a subset of niche cell population (Ding, Saunders et al. 2012, Ding and Morrison 2013, Zhou, Ding et

al. 2015). However, these animal models are unable to recapitulate the unique human BM microenvironment and its influence on hematopoiesis. Accordingly, it is essential to develop new experimental system that could mimic human BM microenvironment.

Previously many studies have used niche cells to establish co-culture systems to study the cell-cell interaction between HSCs and the feeder cells. The co-culture with BM stromal cells, osteoblasts, and endothelial cells increased HSC proliferation while inducing multi-lineage differentiation with some lineage preference (Fraser, Szilvassy et al. 1992, Calvi, Adams et al. 2003, Friel, Heberlein et al. 2005, Jing, Fonseca et al. 2010, Yang, Ma et al. 2013, Huang, Li et al. 2016). As the BM is a 3D (three dimensional) tissue, 3D culture systems are becoming more popular in engineering hematopoietic niches *in vitro*. However, as biophysical properties including the viscosity and ECM composition of BM microenvironment might impact HSC fate decision (Schepers, Campbell et al. 2015), an ideal scaffold should have the same mechanical properties of the BM microenvironment. There are few types of 3D scaffolds that have been explored for HSCs *ex vivo* culture, including porous matrix, nanofiber meshes, woven and non-woven fabrics (Ehring, Biber et al. 2003, Chua, Chai et al. 2007, Mortera-Blanco, Mantalaris et al. 2011, Ferreira, Jahnen-Dechent et al. 2012). These scaffolds provide biological compatibility for cells to survive, enough space for the cells to adhere, and appropriate pore sizes for cells to migrate and exchange nutrients. Some studies have used collagen coated substrates to mimic the 3D soft marrow, and it has been shown to change the shape, spread, and phenotype of HSCs (Mortera-Blanco, Mantalaris et al.

2011, Choi and Harley 2012). However, these models do not recapitulate the BM microenvironment as these systems lack the complexity of BM ECM, which is composed of many different types of proteins and glycosaminoglycans. Therefore, in this we used a novel natural ECM material, DWJM from umbilical cord, as a scaffold to establish a 3D *in vitro* model system to study UCBCD34+ HSPCs fate decision in response to different niche components.

Materials and methods

1. Enrichment of UCB CD34+ HSPCs

Fresh UCB units were obtained from St. Louis cord blood bank (St. Louis, MO, USA). Mononuclear cells were separated by using Lymphoprep (Stemcell Technologies, Canada) density medium followed by immunomagnetic separation of $CD34^+$ cells using the EasySep™ Human Cord Blood CD34 Positive Selection Kit (STEMCELL Technologies, Canada) according to manufacturer's protocol. Briefly, blood was diluted 1:1 in PBS with 2% FBS, and layered on top of density medium in SepMate tubes (STEMCELL Technologies, Canada). After centrifugation, mononuclear cells in supernatant were centrifuged, washed twice to remove platelets, resuspended in 14ml tubes and counted. 1x RBC lysis buffer (STEMCELL Technologies, Canada) was added 1:1 into the cell suspension, followed by adding nanoparticles recognizing cell surface antigen. Tubes were placed in magnet (STEMCELL Technologies, Canada) for 5 minutes, and then supernatant was poured off. PBS with 2% FBS was added to wash the cells and the magnetic step was repeated four times. Enriched cells were centrifuged,

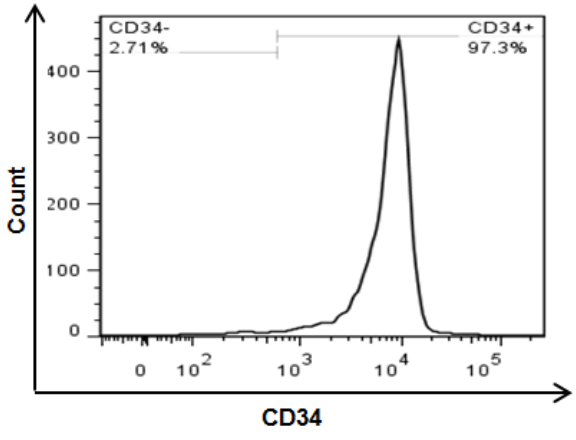
counted, and cell purity was measured by flow cytometry. UCB units with more than 90% purity were used in our experiments (Fig 1A). $CD34^+$ cells were cultured in StemSpan serum-free Medium (Stemcell Technologies, Canada) supplemented with a dilution of 1:100 cytokine cocktail StemSpan cc110 (Stemcell Technologies, Canada) including recombinant human Flt-3 Ligand, Stem Cell Factor, and Thrombopoietin.

2. Isolation of bone marrow MSCs

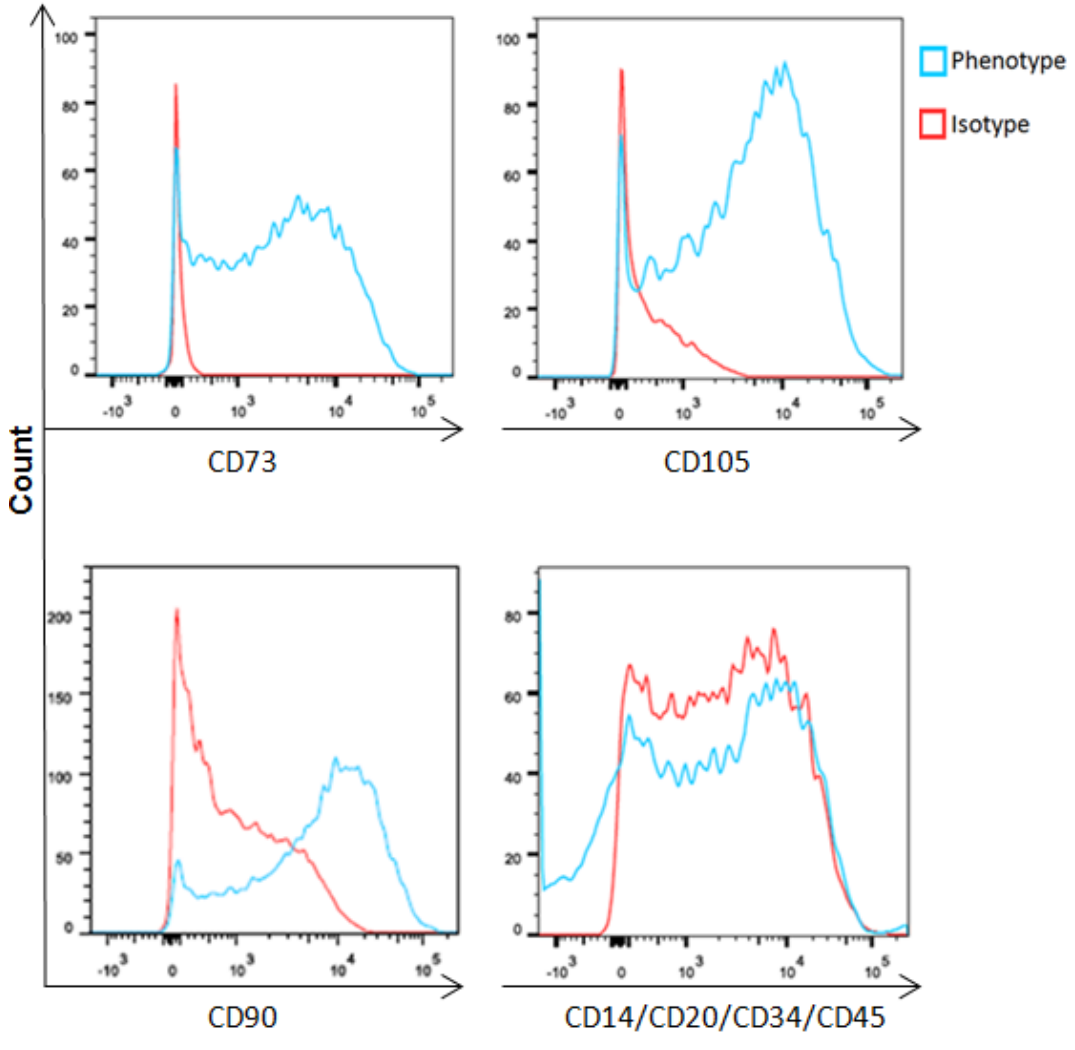
Human bone marrow stromal cells (MSCs) were isolated from bone marrow aspirates collected from healthy donors at the University of Kansas Medical Center (HSC # 5929). The mononuclear cells from bone marrow aspirates were separated by using Lymphoprep (Stemcell Technologies, Canada) density medium according to manufacturer's instructions. Briefly, bone marrow aspirated were diluted 1:1 in PBS, and layered on top of density medium in SepMate tubes (STEMCELL Technologies, Canada), washed, and centrifuged to form pellets. After isolation, cells were counted, and cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM) with 20% FBS and 1% pen/strep at 37°C with 5% CO_2 overnight in T75 culture flasks. The next day, non-adherent cells were removed, and the adherent cells were cultured until use. A MSC phenotyping kit (Miltenyi, Germany) was used to characterize adherent cells. Cells

Figure 1.Characterization of the hematopoietic niche components. (A) Freshly enriched umbilical cord blood (UCB) CD34+ HSPCs with more than 90% purity and (B) bone marrow stromal cells with mesenchymal stem cell (MSC) phenotype CD73+CD105+CD90+CD14-CD20-CD34-CD45- were used to establish the 3D hematopoietic niche cell components.

A


CD34-
2.71%
CD34+
97.3%
Count
CD34

B


Phenotype
Isotype
Count
CD73
CD105
CD90
CD14/CD20/CD34/CD45

positive for CD73, CD90, CD105, and negative for the hematopoietic cells markers CD14, CD20, CD34, and CD45 were used in our study (Fig 1B). The decellularization procedure was described in our recent publication (Aljitawi, Xiao et al. 2013). Briefly, the surrounding membranes and vascular structures were removed followed by dissecting the umbilical cords into large pieces that were subjected to two cycles of osmotic shock followed by an anionic detergent (Sodium lauryl) and, sodium succinate (Sigma L5777), alternating with a recombinant nucleic acid enzyme (Benzonase™), and followed by organic solvent extraction with 40% ethyl alcohol. Next, all of the detergent and other processing residuals were removed by ion exchange beads in a reciprocating flow-through glass system for 30 hours in ddH_2O at room temperature. The decellularized matrix was cryopreserved using 10% human recombinant albumin (Novozymes) and 10% DMSO (Sigma) solution in RPMI 1640 media, employing a material specific computer controlled freezing profile that was developed to freeze at -1°C/minute to -180°C.

3. Decellularization of Wharton's jelly matrix

The decellularization procedure was described in our recent publication (Aljitawi, Xiao et al. 2013). Briefly, the surrounding membranes and vascular structures were removed followed by dissecting the umbilical cords into large pieces that were subjected to two cycles of osmotic shock followed by an anionic detergent (Sodium lauryl) and, sodium succinate (Sigma L5777), alternating with a recombinant nucleic acid enzyme (Benzonase™), and followed by organic solvent extraction with 40% ethyl alcohol. Next,

all of the detergent and other processing residuals were removed by ion exchange beads in a reciprocating flow-through glass system for 30 hours in ddH_2O at room temperature. The decellularized matrix was cryopreserved using 10% human recombinant albumin (Novozymes) and 10% DMSO (Sigma) solution in RPMI 1640 media, employing a material specific computer controlled freezing profile that was developed to freeze at -1°C/minute to -180°C.

4. Preparation of *in vitro* bone marrow mimetic model

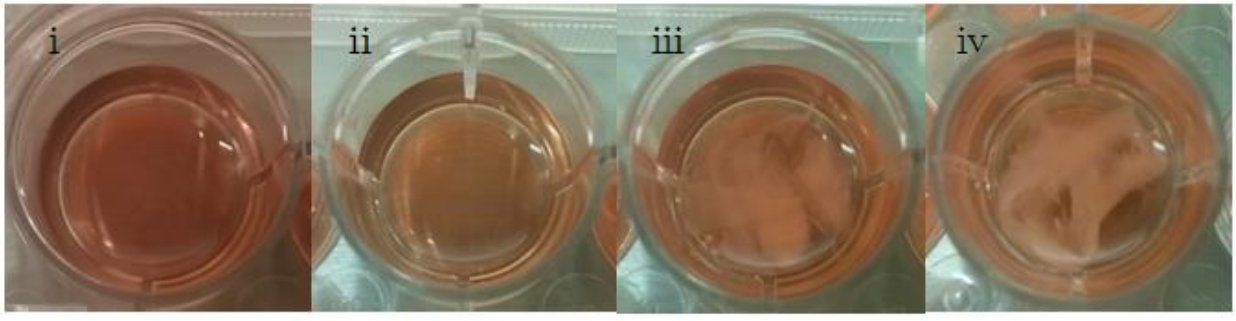
Enriched CD34+ cells were cultured in cytokine alone as suspension cells, or as an adherent cells co-cultured with bone marrow stromal cells forming a monolayer in with bone marrow MSCs. Before seeding cells into DWJM, DWJM scaffolds were washed in PBS three times, and incubated in culture medium overnight. For co-culture, MSC were seeded into culture wells alone as monolayers or into DWJM as 3D systems two days before adding $CD34^+$ cells. After enrichment, $3x10^4$ $CD34^+$ cells were seeded (i) in suspension without MSCs, (ii) on a monolayer of MSC, (iii) in cell-free DWJM and (iv) in DWJM with pre-seeded bone marrow stromal cells (Fig 2A). The three-dimensional culture systems are divided into two compartments: $CD34^+$ growing (1) in suspension around DWJM and (2) inside DWJM with strong adhesion (Fig 2B). Cells were cultured for 7 days at 37°C with 5% CO_2.

5. CellTrace proliferation assay

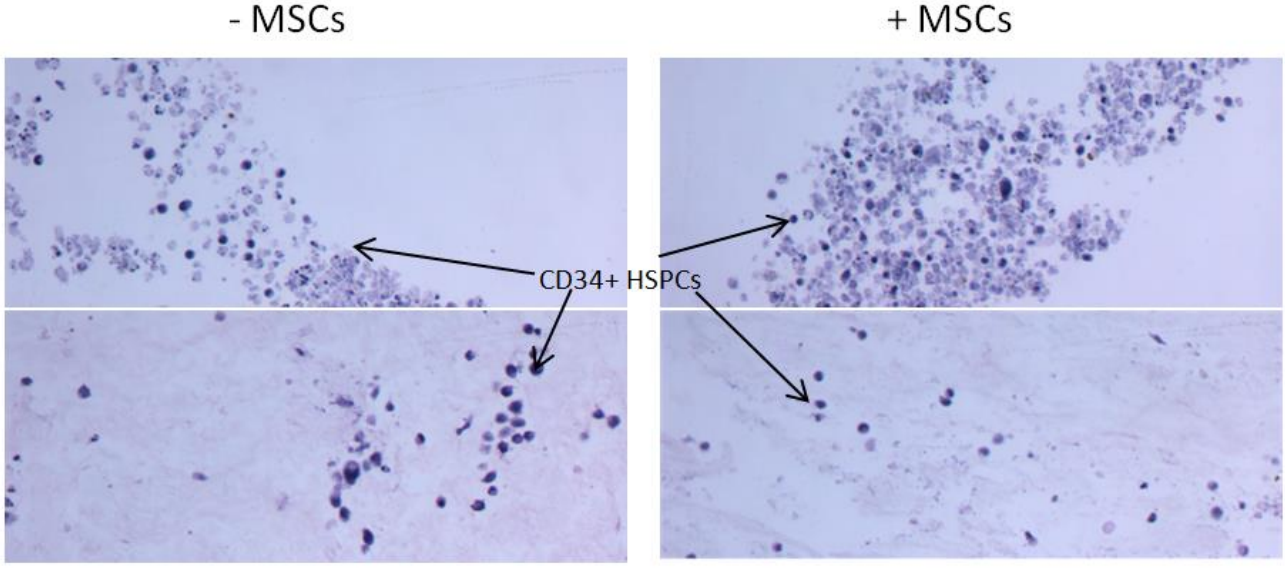
Cell division was monitored by using CellTrace proliferation kit (Invitrogen, USA). The stable violet fluorescent dye can bind to the amines in the cells covalently, and

Figure 2. Preparation of *in vitro* bone marrow mimetic model. (A) UCB HSPCs were cultured in culture wells either: i) in suspension (control) ii) Over a BM-MSC monolayer iii) with DWJM and iv) with DWJM preseeded with BM-MSCs and cultured for 7 days in the presence of cytokine supplementation. (B) Histology H&E stained sections of the two compartments (Suspension (upper) vs Adherent (bottom)) of 3D DWJM-based culture systems. Hematopoietic cells were identified in DWJM (Left) and DWJM preseeded with BM-MSCs (Right) respectively after 7 days culture (20x).

A



B



distributed into two daughter cells equally after each cell division. Soon after enrichment, $CD34^+$ cells were labelled with violet dye at a concentration of 1 µl reagent per 10^6 cells suspended in 1 ml PBS, incubated for 20 minutes at room temperature. The reaction was stopped by adding PBS with 10% FBS after 5 minutes incubation followed by washing with PBS.

The cell proliferation was measured by flow cytometry soon after labeling and 7 days after culture. A CD34-FITC antibody (clone AC136, Miltenyi, Germany) was used for co-staining before flow cytometry analysis to label $CD34^+$ cells. Data was analyzed by using Flow Jo software (Version 7.5, Oregon, USA).

6. AnnexinV / PI apoptosis assay

Hematopoietic cells were stained with annexin V-FITC, according to manufacturer's instructions (FITC Annexin V Apoptosis Detection Kit II, BD Pharmingen, USA). Annexin V binds to cells apoptotic cells, and PI is to distinguish viable cells from nonviable cells. Briefly, cells were washed with cold PBS, resuspended in 1x staining buffer. Then 5µl of annexin V-FITC and 10µl of PI reagents were added into up to 10^5 cells resuspended in 100µl staining buffer, incubated for 15 minutes at room temperature, and analyzed by flow cytometry within 1 h. A CD34-APC antibody (Clone AC136, Miltenyi, Germany) was used to co-stain with these reagents before flow cytometry analysis to label CD34+ cells. Data were analyzed by using Flow Jo software.

7. Analysis of surface-markers by flow cytometry

The phenotype of cultured hematopoietic cells was examined 7 days after seeding. PBS+2% FBS was used as staining buffer. Cells were washed with staining buffer twice, resuspended, and stained by FcR blocking Reagent (Miltenyi, Germany) for 10 min at 4 °C followed by staining with CD34-FITC (Clone AC136, Miltenyi, Germany), CD38-APC (clone IB6, Miltenyi, Germany), CXCR4-PE-Vio770 (clone 12G5, Miltenyi, Germany), c-kit-APC-Cy7 (clone 12G5, Miltenyi, Germany), CD41-FITC (clone HIP8, STEMCELL Technologies, Canada), CD71-VioBlue (clone AC102, Miltenyi, Germany), CD33-PE(WM53, Biolegend, USA), CD3-FITC (clone UCHT1, STEMCELL Technologies, Canada), CD19-APC (clone HIB19, BD Pharmingen, USA), and CD56-PE-Cy7 (clone B159, BD Pharmingen, USA) for 10 min at 4°C. After staining, cells were washed and resuspended in 400 µl staining buffer and analyzed by flow cytometry (LSR II, BD Biosiences). Data were analyzed using Flow Jo software.

8. Colony forming unit (CFU) assay

The clonogenic ability of cells cultured in different conditions was measured by CFU assay. After 7 days culture, cells were harvested, and washed in PBS as described above. Then 300 cells were added in triplicate in 1 ml of MethoCult methylcellulose medium supplemented with recombinant human SCF, G-CSF, GM-CSF, IL3 and erythropoietin (STEMCELL Technologies, Canada), which allows the growth of erythroid progenitors (CFU-E and BFU-E, Fig 3A), granulocyte-macrophage progenitors (CFU-GM, Fig 3B) and multi-potent granulocyte, erythroid, macrophage, megakaryocyte progenitors (CFU-GEMM, Fig 3C). Samples were plated in triplicate in 35 mm^2 tissue

culture dishes and incubated at 37°C with 5% CO_2. Colonies consisting of >30 cells were counted using an inverted microscope 12–14 days after plating.

9. Transmigration assay

The transmigration assay was used to evaluate the chemotactic responses of the cells to stromal cell-derived factor-1 (SDF-1) after 7 days culture. It was a two-compartment chamber (Corning Costar, New York, NY, USA) setting: in the upper compartment, up to $8x10^4$ cells in 400ul RPMI1640 with 10% FBS were seeded after harvested from each culture condition; while in the bottom compartment, 600 μl of RPMI1640 with 10% FBS and 125 ng/mL SDF-1was added. Triplicate wells were set up for each group. For each experimental set, the bottom compartment of two wells were filled with RPMI1640 with 10% FBS but without SDF-1 in order to assess spontaneous migration. Cells were incubated at 37°C with 5% CO_2 for 4 hours. Then the upper compartment was removed and the migrated cells in the lower compartment were harvested and counted.

10. Statistical analysis

Data is presented as mean ± standard error of the mean (SEM). Comparisons between different culture conditions were done by using Student's t test. For all analyses, $P<0.05$ was considered statistically significant. Analyses were performed using GraphPad Prism 6 (GraphPad Software Inc, USA).

Results

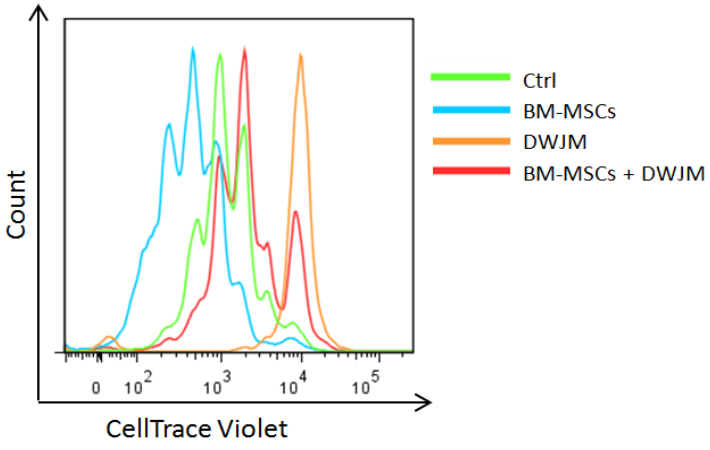
DWJM slowed UCB CD34$^+$ HSPC proliferation while maintaining its primitive phenotype.

We began with assessing HSPCs proliferation under our different culture conditions. First we counted cell numbers by trypan blue exclusion. We found that culture conditions that included stromal cells showed enhanced proliferation of hematopoietic cells over non-stromal cell conditions, while UCB CD34$^+$ cells cultured in DWJM alone showed slowed proliferation (Fig 3B). Among all of the four culture conditions, stromal cell mono-layer showed the highest yield with a 50-fold increase compared to 26-fold in control. The lowest overall proliferation was observed in UCB CD34$^+$ cells cultured in DWJM without stromal cell support, reaching a 7-fold increase. Although pre-seeding of stromal cells in DWJM supported the proliferation of UCB CD34$^+$ cells by increasing cell numbers by approximately 15-fold, the proliferation was still slower than the control group.

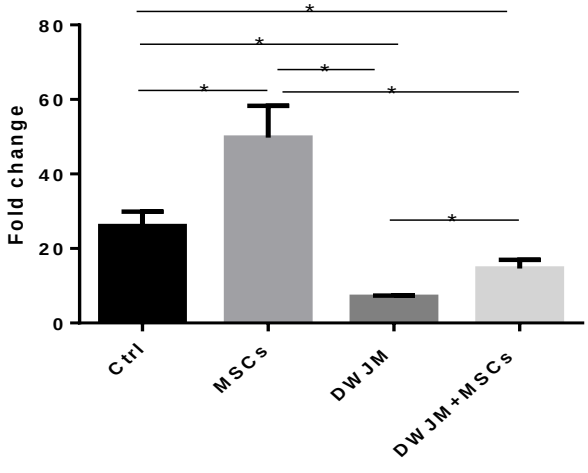
To further assess proliferation of UCB CD34$^+$ cells, we tracked cell division by using CellTrace assay. Consistent with fold change in cell number data using trypan blue, bone marrow stromal cell mono-layer supported HSPC proliferation the most, as is shown by the most significant loss of violet signal. Cells undergoing 4~6 cell divisions were significantly increased compared to the other culture conditions. In contrast, UCD CD34$^+$ cells cultured in DWJM alone did not show enhanced proliferation, as most of the cells

Figure 3. DWJM inhibited UCB HSPC proliferation while maintaining CD34+ phenotype in the majority of cells. (A) Representative histograms of CellTrace Violet intensity of UCB HSPC cultured on DWJM scaffolds with and without BM MSC support after 7 days of culture. (B) Cell number fold change of hematopoietic cells quantified using hematocytometer according to culture conditions (n = 3, data are expressed as mean ± SEM, $^{*}P < 0.05$). (C) Representative CellTrace Violet/CD34 co-staining of cells according to culture conditions.

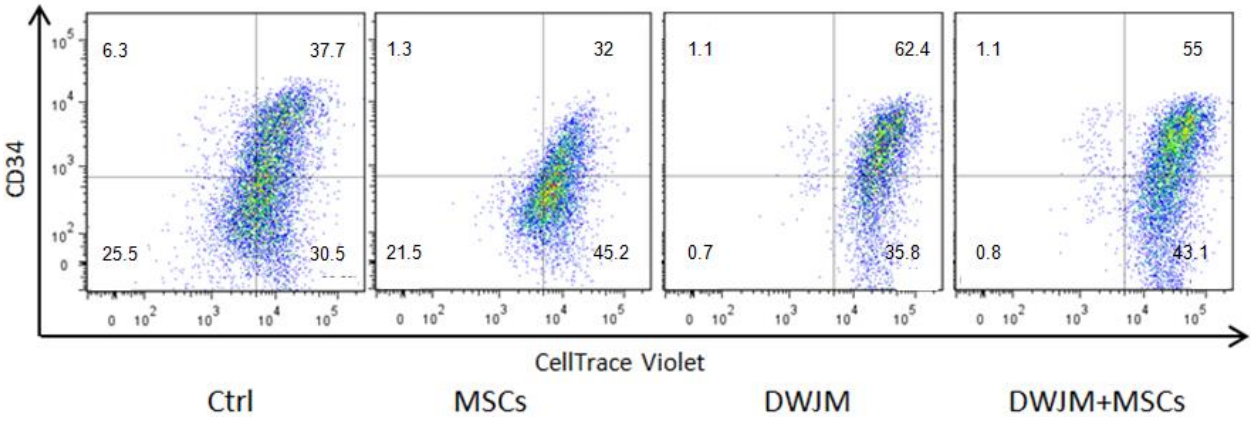
A


Count
CellTrace Violet
Ctrl
BM-MSCs
DWJM
BM-MSCs + DWJM

B


Fold change
Ctrl
MSCs
DWJM
DWJM+MSCs

C


CD34
CellTrace Violet
6.3
37.7
25.5
30.5
1.3
32
21.5
45.2
1.1
62.4
0.7
35.8
1.1
55
0.8
43.1
Ctrl
MSCs
DWJM
DWJM+MSCs

maintained an extremely high violet fluorescence with only a small proportion undergoing cell division. DWJM preseeded with stromal cells increased UCB $CD34^+$ proliferation,since there were some cells undergoing 1~3 cell divisions, and the pattern of signal loss resembled that of cells cultured in control condition (Fig 3A).

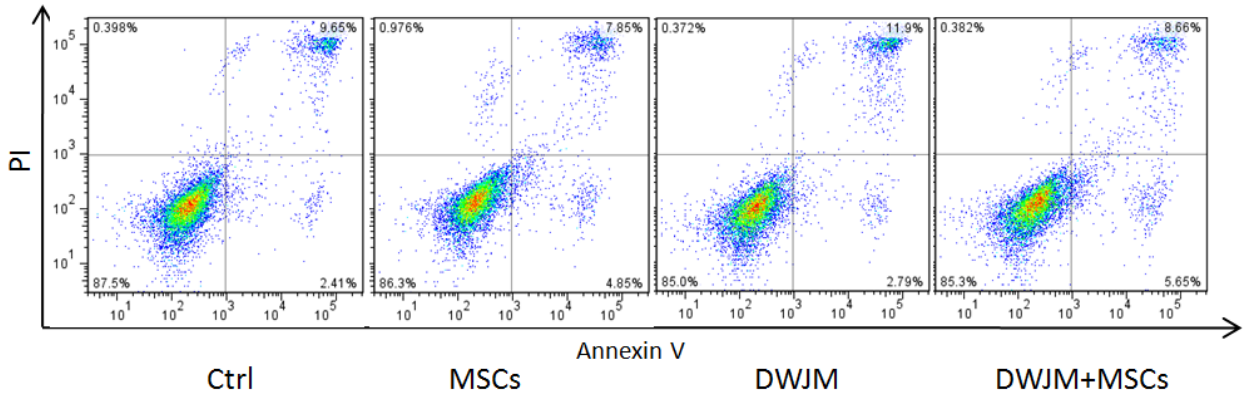
Since increased proliferation of HSPCs is usually at the expense of their differentiation, we next analyzed the ability of the cultured cells to maintain their primitive phenotype while undergoing proliferation by co-staining with CD34 antibody (Fig 3C). We defined fast-proliferating and slow-proliferating cells according to violet fluorescence intensity. In stromal cell mono-layer culture condition, almost all fast-proliferating cells were $CD34^-$, while in the control condition, a small portion of fast proliferating cell were $CD34^-$. In contrast, almost all the cells in DWJM-based 3D culture conditions were slow-proliferating, and they maintained the highest frequency of $CD34^+$ expression. Thus DWJM alone sustained a more quiescent $CD34^+$ cells.

DWJM maintains UCB CD34+ HSPCs viability

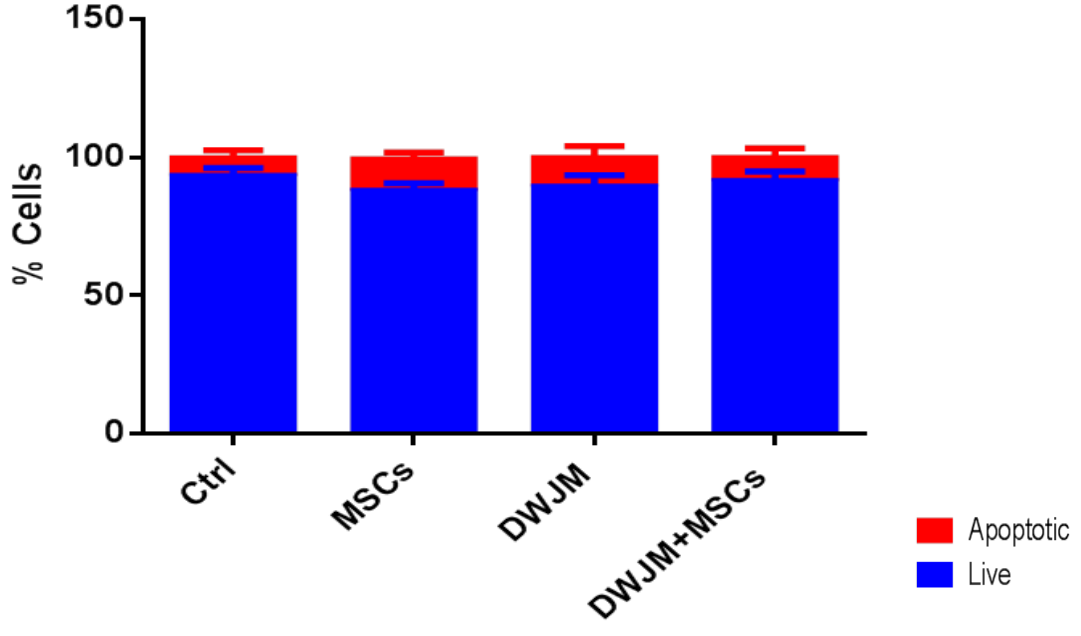
Apoptosis plays a critical role in the maintenance of HSC pool as well as lineage committed cells. One function of the engineered *in vitro* bone marrow microenvironment is to keep the vitality of stem cells, progenitors, and mature cells. To evalute apoptosis in cells cultured in each condition, we performed Annexin V / PI analysis which distinguishes between live (Annexin V^-PI^-), early apoptotic (Annexin V^+PI^-), late apoptotic (Annexin V^+PI^+), and necrotic (Annexin V^-PI^+) cells. We found that rate of apoptosis did not change among all the culture conditions ranging from 80%~95%, and necrosis was

Figure 4. DWJM maintains UCB CD34+ HSPCs viability. Apoptosis and necrosis of hematopoietic cells cultured according to culture conditions by co-staining of AnnexinV/PI and flow cytometry analysis. Results are shown as (A) density plots and (B) quantification (*n* = 3, data are expressed as mean ± SEM)

A


PI
Annexin V
0.398%
9.65%
87.5%
2.41%
0.976%
7.85%
86.3%
4.85%
0.372%
11.9%
85.0%
2.79%
0.382%
8.66%
85.3%
5.65%
Ctrl
MSCs
DWJM
DWJM+MSCs

B


% Cells
150
100
50
0
Ctrl
MSCs
DWJM
DWJM+MSCs
Apoptotic
Live

negligible in every condition, indicating a normal cell turnover but no enhanced apoptosis (Fig 4 A and B). To further evaluate apoptosis of HSPCs after 7 days culture, we co-stained cells with CD34 antibody. The apoptosis rate of $CD34^+$ cells was similar across different culture conditions (data not shown).

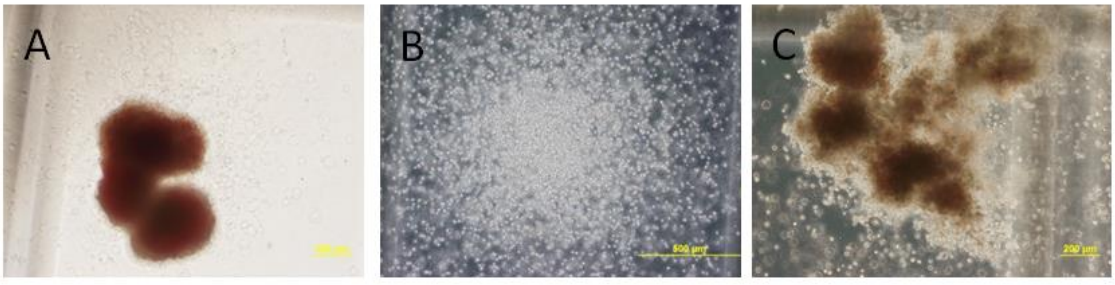
DWJM enhances clonogenic potential of UCB CD34+ HSPCs

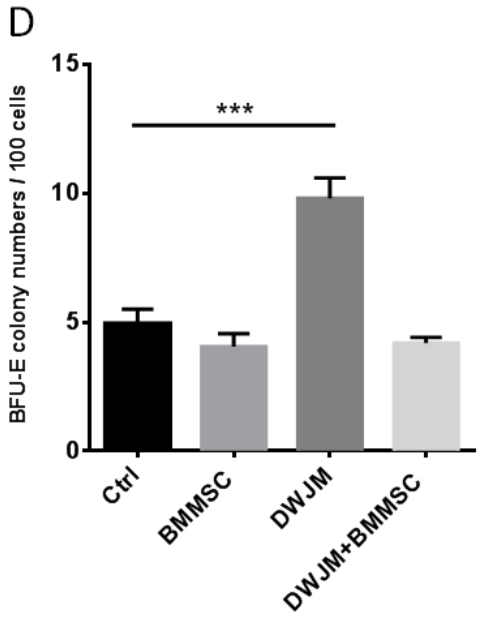
Since proliferation is usually associated with differentiation, we performed colony forming assay, a functional assay to evaluate the frequency of expanded HSPCs with clonogenic ability, to test the capacity of different culture systems to maintain HSPC functions. Compared to control, HSPCs cultured in DWJM alone demonstrated approximately 2-fold increase in all the BFU-E ($p<0.0001$), CFU-GM ($P<0.01$), and CFU-total colony formation ($P<0.01$), indicating that DWJM material maintained self-renewal and prevented fast differentiation of HSPCs. The pre-seeding of DWJM with bone marrow stromal cells also gave rise to a significant increase of CFU-GM, but not BFU-E, suggesting that the stromal cells embedded in DWJM might lead to myeloid differentiation at the expense of erythroid differentiation. Bone marrow stromal cells alone in monolayer did not have any influence on the clonogenic potential compared to control (Fig 5).

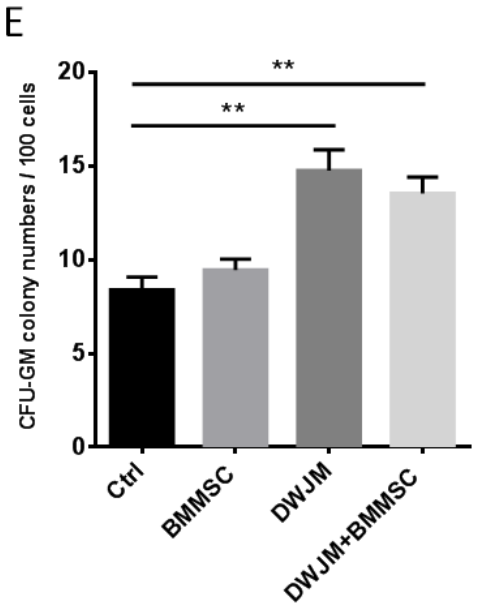
DWJM maintains UCB CD34+ HSPCs in their primitive phenotype

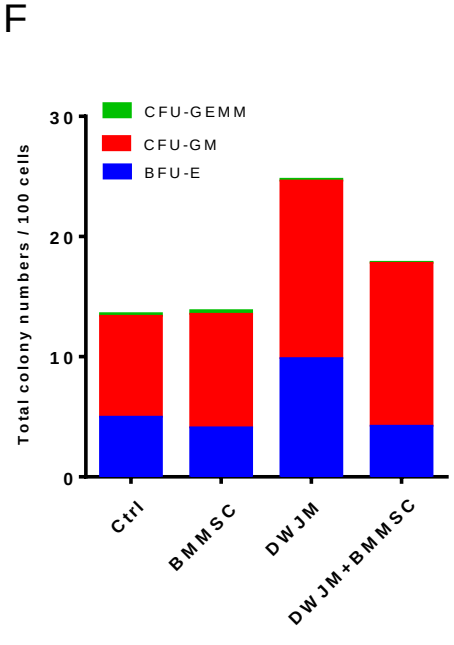
We next examined the effects of different culture conditions on maintenance of the primitive phenotypes of HSPCs, since one main function of stem cell niche is to keep the

Figure 5. DWJM increases clonogenic ability of HSPCs. (A-C) Representative pictures of (A) BFU-E, (B) CFU-GM, and (C) CFU-GEMM observed by using an inverted microscope. (D-F) Numbers of (D) BFU-E, (E) CFU-GM, and (F) total colonies per 100 seeded cells generated from different culture conditions. (n = 3, data are expressed as mean ± SEM, $^{**}P < 0.01$, $^{***}P < 0.001$).


A
B
C


D
BFU-E colony numbers / 100 cells
15
10
5
0
***
Ctrl
BMMSC
DWJM
DWJM+BMMSC


E
CFU-GM colony numbers / 100 cells
20
15
10
5
0
**
**
Ctrl
BMMSC
DWJM
DWJM+BMMSC

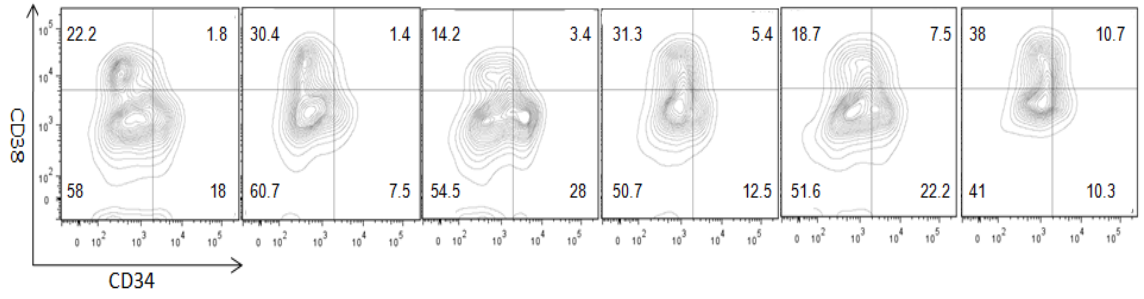

F
CFU-GEMM
CFU-GM
BFU-E
Total colony numbers / 100 cells
30
20
10
0
Ctrl
BMMSC
DWJM
DWJM+BMMSC

pool of HSCs. To elucidate whether there will be some changes due to the HSPCs adhesion to DWJM or MSCs, as we saw some cells migrated into and embedded within DWJM (Fig 2B), we analyzed the UCB $CD34^+$ cells in both compartments; suspension compartment where cells are floating in the culture well or loosely attached to DWJM; and adherent compartment where cells are embedded in DWJM (Fig 2B). We first compared the maintenance of $CD34^+$ phenotype, which is an indicator of the HSPC population used for stem cell transplantation. The percentage of $CD34^+$ cells was the lowest in co-culture with MSC monolayer. DWJM preseeded with stromal cells recovered the $CD34^+$ phenotype compared to the stromal cell monolayer. UCB $CD34^+$ cells cultured in DWJM, regardless of adherent or not, contained a higher frequency of $CD34^+$ HSPCs, although the absolute increase might not be biological important (Fig 6A). We further analyzed the more primitive $CD34^+CD38^-$ HSC population (Fig 6A). All culture conditions with bone marrow MSCs gave significantly lower frequency of CD34+CD38- cells, compared to their counterparts without stromal cells. DWJM maintained the $CD34^+CD38^-$ population at a similar rate to control. In general, bone marrow stromal cells stimulated the differentiation of HSCs, while DWJM alone had minimal effects on HSC phenotype.

Since we saw stem cell phenotype and functional changes, we sought to find out a potential mechanism giving rise to those changes. C-Kit, the ligand of stem cell factor (SCF), is highly expressed in cord blood CD34+ cells (data not shown). SCF also promotes HSC self-renewal and proliferation in vitro culture along with other cytokines

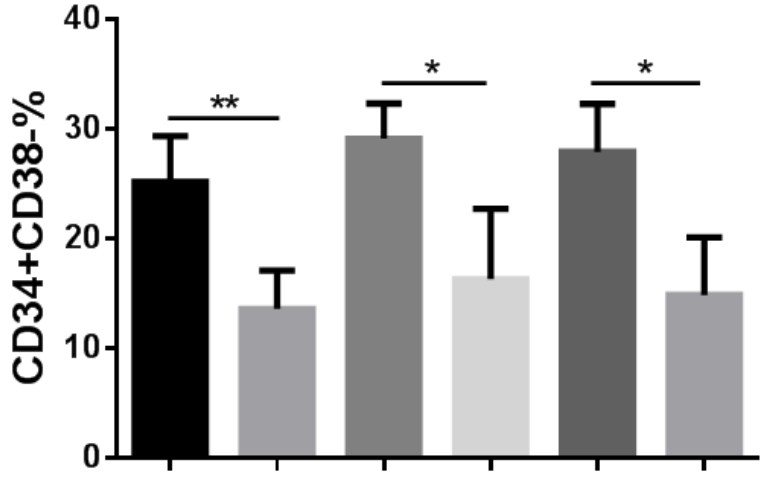
Figure 6. DWJM maintains CD34+CD38- phenotype while enhancing c-kit expression in cultured UCB HSPCs. (A) Representative contour plot and (B) quantification of the primitive CD34+CD38- phenotype of hematopoietic cells according to different culture conditions. (C) Flow cytometry scheme used to analyze c-kit expression in hematopoietic cells according to different culture conditions. Cells were stained with antibodies against CD34, CD38, and c-kit. Then the c-kit+ cell percentages were analyzed within CD34+ HSPCs, CD34+CD38- HSCs/MPPs, and CD34+CD38+ HPCs. (D) Quantification of the c-kit expression in hematopoietic cells cultured on DWJM scaffolds with and without stromal support after 7 days (n = 3, data are expressed as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$)

A



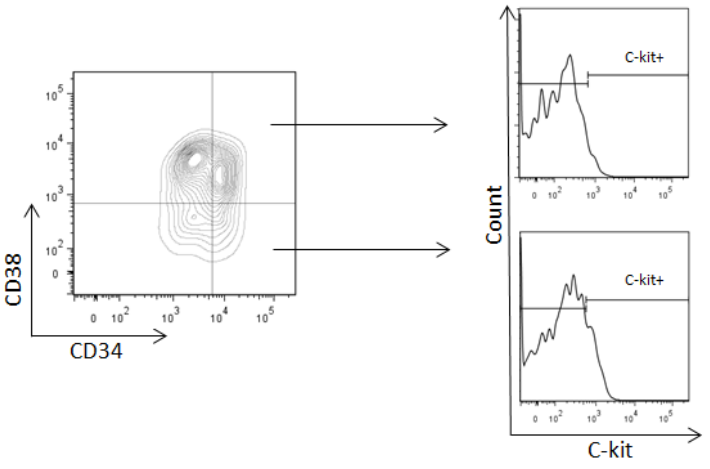
MSCs	-	+	-	+	-	+
DWJM	-	-	+	+	+	+
Adhesion MSC/DWJM	-/-	+/-	-/-	-/-	-/+	+/+

B

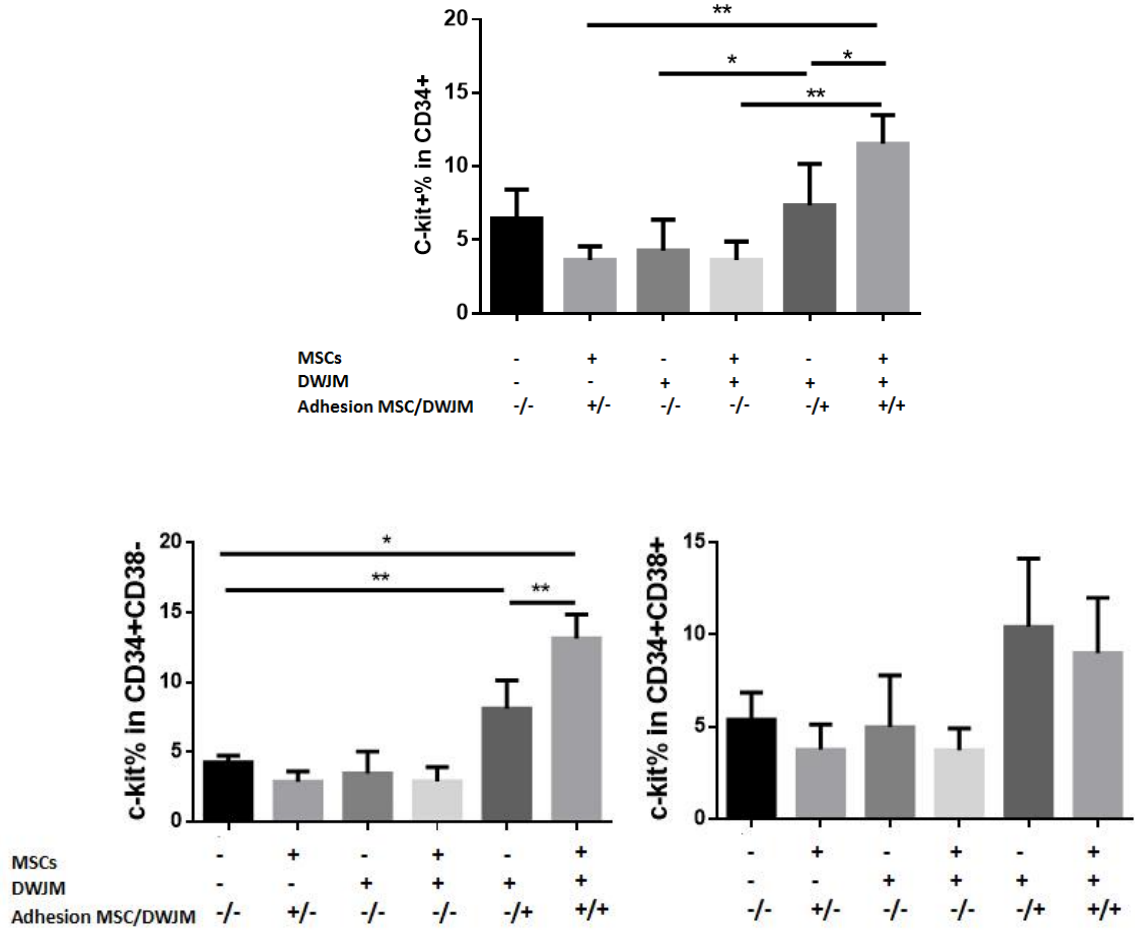


MSCs	-	+	-	+	-	+
DWJM	-	-	+	+	+	+
Adhesion MSC/DWJM	-/-	+/-	-/-	-/-	-/+	+/+

C


CD38
CD34
C-kit+
C-kit+
Count
C-kit

D


C-kit+% in CD34+
MSCs - + - + - +
DWJM - - + + + +
Adhesion MSC/DWJM -/- +/- -/- -/- -/+ +/+
c-kit% in CD34+CD38-
c-kit% in CD34+CD38+
MSCs - + - + - +
DWJM - - + + + +
Adhesion MSC/DWJM -/- +/- -/- -/- -/+ +/+

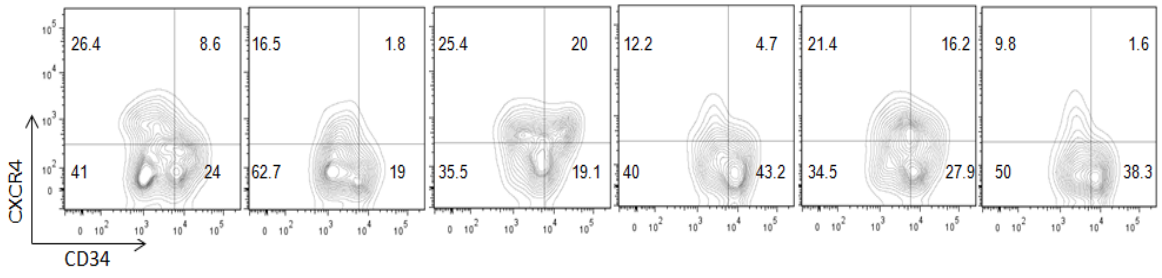
(Zandstra, Conneally et al. 1997). After culture, the $CD34^+$c-Kit^+ population were seen at a low frequency in all the culture conditions (1%~5%, data not shown). Although not statistically significant, HSPCs adherence to DWJM increased c-Kit expression, especially in DWJM preseeded with stromal cells (Fig 6D). We further analyzed c-Kit^+ population within $CD34^+CD38^-$ HSCs/MPPs and $CD34^+CD38^+$ oligopotent progenitors (Fig 6C). Within the $CD34^+CD38^-$ population, the adhesion to DWJM alone enhanced c-Kit+ population by 1.5-fold, while DWJM pre-seeded with stromal cells enhanced c-kit+ cells by 2.5-fold, compared to control. However within $CD34^+CD38^+$ population, there was no significant change in c-Kit expression in response to adhesion (Fig 6D).

DWJM increases UCB $CD34^+$ HSPC CXCR4 dependent migration toward SDF-1

One challenge for UCB transplantation is delayed engraftment partially because of impaired bone marrow homing. CXCR4/SDF-1 axis plays an important role in homing of HSC to bone marrow post-transplantation. CXCR4 is the receptor of chemokine SDF-1 abundant in bone marrow microenvironment. We found that in freshly isolated $CD34^+$ cells, the CXCR4 expression is extremely low, usually ranging from 6% to 8% (6 units tested). Under our tested culture conditions, DWJM suspension compartment induced CXCR4 expression the most, both in the total mononuclear cells and $CD34^+$ HSPCs (Fig 7 A and B). The adhesion to DWJM did not have any significant effect on CXCR4 expression. In the presence of bone marrow stromal cells, CXCR4 expression was significantly reduced in both 2D and 3D systems ($p< 0.0001$ and $p< 0.001$). We

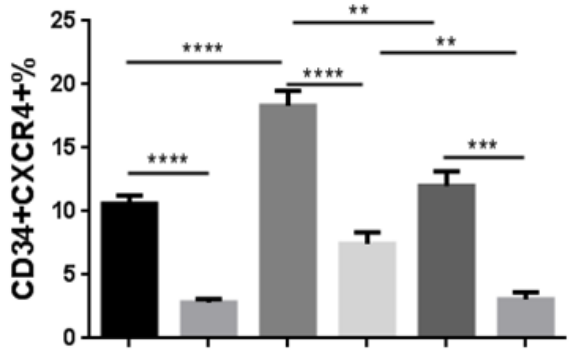
Figure 7. CXCR4/CD34+ expression is reduced in the presence of MSCs and is significantly increased in the non-adherence population of cells cultured with DWJM. (A) Representative contour plot and (B) quantification of the CXCR4 expression in hematopoietic cells according to different culture conditions (n = 3, data are expressed as mean ± SEM, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$). (C) The migration abilities depending on CXCR4/SDF-1 axis of the expanded cells were assessed by a transwell assay (n = 3, data are expressed as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$).

A



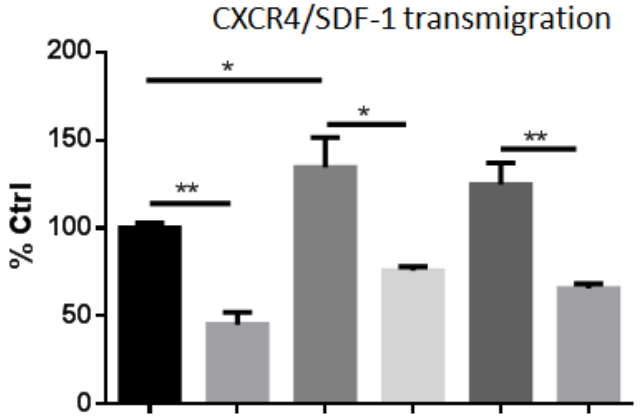
MSCs	-	+	+	+	+	+
DWJM	-	-	-	+	-	-
Adhesion MSC/DWJM	-/-	+/-	-/-	-/-	-/+	+/+

B



MSCs	-	+	-	+	-	+
DWJM	-	-	+	+	+	+
Adhesion MSC/DWJM	-/-	+/-	-/-	-/-	-/+	+/+

C



MSCs	-	+	-	+	-	+
DWJM	-	-	+	+	+	+
Adhesion MSC/DWJM	-/-	+/-	-/-	-/-	-/+	+/+

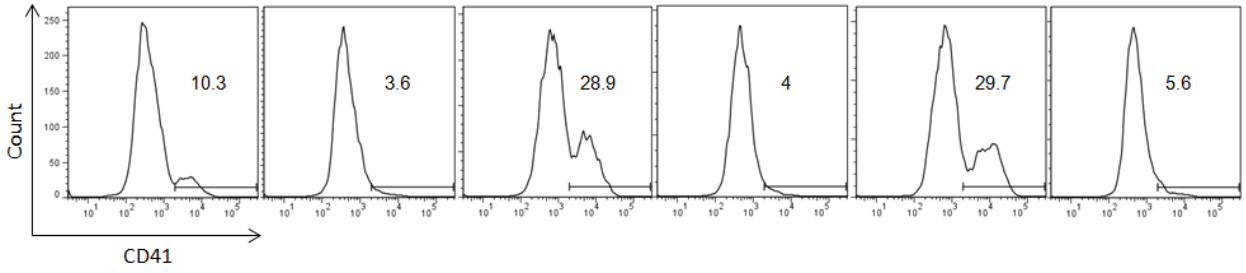
subsequently applied transmigration assay to further assess the influence of DWJM and stromal cells on the homing ability of the cells from different culture conditions based on CXCR4/SDF-1 axis (Fig 7C). The results were consistent with CXCR4 expression findings: UCB CD34+ cells cultured with stromal cells demonstrated decreased migration to SDF-1, while UCB $CD34^+$ cells cultured in the suspension compartment of DWJM showed a slight (up to 50%) but statistically ($p< 0.05$) significant increased migration rate. However the migration rate of the cells in the adhesion compartment of the 3D culture systems did not show any changes compared to their non-adhesion counterparts.

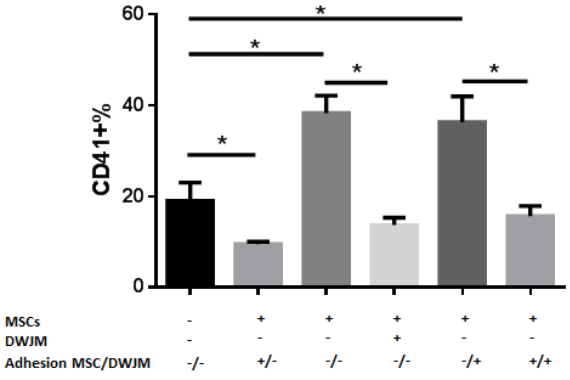
DWJM promotes megakaryocyte differentiation of UCB CD34+ HSCPs

Since HSPCs in different culture conditions underwent various levels of differentiation, to obtain a detailed view of the fate of the HSPCs, we analyzed the frequency of all the hematopoietic lineages by staining cells with lineage specific markers. Within the lymphoid lineage, $CD19^+$ B cells were extremely rare in every culture condition and were hardly detected by flow cytometry (all less than 0.5%). $CD3^+$ T cell frequency was also very low (less than 10%), and no significant differences were seen among all culture conditions. CD33, a myeloid specific marker is highly expressed in hematopoietic cells cultured in all compartments, ranging from 80% to 98% (Fig 8B), indicating that our culture conditions are more prone to myeloid differentiation. We also found that there were no significant differences in $CD71^+$ erythroblast differentiation as well as $CD56^+$ natural killer (NK) cell differentiation (Fig 8B). However as almost all the cells are also

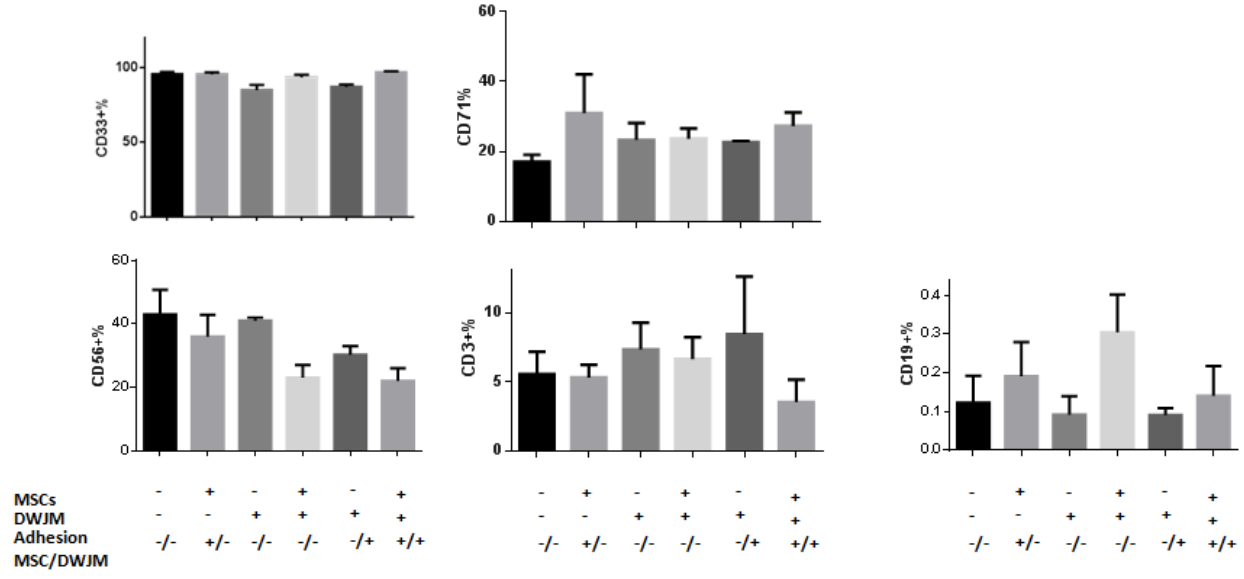
Figure 8. DWJM enhanced megakaryocyte differentiation. (A) Representative histograms and quantification of CD41+ frequency and (B) quantification of other lineage differentiation markers in the hematopoietic cells according to different culture conditions. The cells were analyzed for the myeloid differentiation (CD33+), megakaryocyte differentiation (CD41+), erythroid differentiation (CD71+), nature killer cell differentiation (CD56+), T cell differentiation (CD3+), and B cell differentiation (CD19+) by flow cytometry.

A


Count
CD41
10.3
3.6
28.9
4
29.7
5.6


CD41+%
MSCs
DWJM
Adhesion MSC/DWJM
-/-
+/-
-/-
-/-
-/+
+/+

B


CD33+%
CD71%
CD56+%
CD3+%
CD19+%
MSCs
DWJM
Adhesion MSC/DWJM
-/-
+/-
-/-
-/-
-/+
+/+

$CD33^+$, the $CD56^+$ NK cells might have been derived from the myeloid rather than lymphoid progenitors.

Finally we measured megakaryocyte differentiation by their surface marker CD41. Generally bone marrow stromal cells suppressed differentiation into megakaryocytes, both in monolayer and DWJM systems. In contrast, we found that there was significantly enhanced $CD41^+$ population within the 2 compartments of DWJM only culture system (Fig 8A).

Discussion

Current efforts focusing on factors affecting HSC fate decisions are mostly based on *in vivo* knock-out animal models (Ding, Saunders et al. 2012, Ding and Morrison 2013, Zhou, Ding et al. 2015). These models are able to identify the key factors in the BM niche but cannot recapitulate the HSC behaviors in human BM (Choi, Mahadik et al. 2015). Therefore an engineered bone marrow would have great clinical significance in studying the biology of hematopoiesis and even leukemogenesis. In addition, these models will help in *ex vivo* expansion of HSCs efforts. During the past years, attempts have been ongoing to engineer an *in vitro* culture system to mimic the bone marrow microenvironment (Choi, Mahadik et al. 2015). Many natural and synthetic biomaterials have been used to mimic the soft marrow mechanics (Lee and Kotov 2009, Lutolf, Doyonnas et al. 2009, Demange, Kassim et al. 2013). Since the impact of ECM on HSC fate decision is only poorly understood, most of the current systems are co-culture of HSCs with a feeder layer of niche cells to support HSC self-renewal and differentiation *in*

vitro. The bone marrow stromal cells are most commonly used niche cell population since they can easily be isolated from donors' bone marrow aspirates. Studies have shown that in the serum-free culture conditions, these stromal feeder layers are able to expand HSPCs (Fraser, Szilvassy et al. 1992, Verfaillie 1992, Jing, Fonseca et al. 2010, Choi and Harley 2012). As the understanding of the niche is growing, the role of endothelial cells and bone-forming osteoblasts in maintaining HSCs are elucidated and co-culture with these cells populations have been shown to support long-term HSC expansion (Rafii, Shapiro et al. 1995, Taichman, Reilly et al. 1996, Chute, Saini et al. 2002, Mishima, Nagai et al. 2010). Besides, they have also been shown to support the differentiation of HSCs to myeloid lineages including erythrocyte (Giarratana, Kobari et al. 2005), megakaryocyte (Hatami, Andrade et al. 2015), monocyte/macrophage (Salati, Lisignoli et al. 2013), as well as lymphoid lineages including natural killer cells (Dezell, Ahn et al. 2012), T cells (Taqvi, Dixit et al. 2006), and B cells (Memon, Feingold et al. 1992).

In this study, we used decellularized Wharton's jelly matrix, the ECM part of umbilical cord, as a scaffold material to in vitro engineer bone marrow hematopoietic niche. We showed that DWJM ECM interactions maintained $CD34^+$ cell primitive phenotype and self-renewal ability. Importantly, DWJM significantly enhanced the transmigration toward SDF-1 of the cultured UCB $CD34^+$ cells by inducing the expression of CXCR4. The HSPCs cultured in DWJM also have multi-lineage differentiation potential, and were especially prone to the megakaryocyte differentiation. All these results indicate that

DWJM could serve as a candidate ECM-based model to support hematopoiesis.

Since DWJM has never been used to culture hematopoietic cells, we first tested whether it was able to support the growth of cord blood $CD34^+$ cells. We first found that DWJM was able to direct a small population of HSPCs migrating into it. MSCs preseeded in the matrix did not increase the cells embedded in DWJM significantly, suggesting that most of the hematopoietic cells prefer to grow in a more liquid environment. We then analyzed the proliferation of UCB $CD34^+$ cells in DWJM, interestingly, cells proliferated more robustly in the suspension compartment with a culture medium, which further increased by adding BM-MSC feeder layer. Thus, adhesion to DWJM is associated with a higher rate of quiescence in our model. Since previous work have shown that in mouse BM, an overwhelming proportion of HSCs are quiescent (Acar, Kocherlakota et al. 2015), the next question in our study was whether the cells were able to maintain their primitive phenotype in DWJM-based culture system. Previous work has proved that enhanced HSPC expansion is at the expense of loss of primitive phenotype and stem cell functions. Thus, we introduced the CellTrace proliferation assay to simultaneously study cell division and the primitive $CD34^+$ phenotype. Consistent with the previous findings, the fast-dividing cells lost the $CD34^+$ phenotype more frequently than the slow-dividing cells, and cells cultured in DWJM based systems had much higher $CD34^+$ rate compared to mono layer culture conditions. In contrast to previous studies (Jang, Jung et al. 2006, Robinson, Ng et al. 2006, Walenda, Bokermann et al. 2011), we found that the fast-dividing cells in BM-MSC monolayer were mostly CD34-, indicating that these

stromal cells strongly promote the differentiation of HSPCs. The presence of of DWJM rescued CD34 expression to some extent.

As the viscosity is an important factor of BM mechanics, and ECM can increase the viscosity by their natural physical properties, previous studies have been done to investigate the relationship between viscosity and HSC maintenance. These studies were mostly based on specific man-made soft-gel matrix with one or several certain known components, making these matrix materials lack of several of the main ECM components in the BM (Feng, Chai et al. 2006, Sagar, Rentala et al. 2006, Leisten, Kramann et al. 2012). Thus, they might not recapitulate the genuine properties of BM ECM. In contrast, DWJM composition resembles the BM; therefore, our model is more appropriate to study the mechanical properties effects on hematopoietic cell proliferation.

An interesting finding of our work is that although this biomaterial did not promote the expansion of HSPCs as fast as the other culture conditions, it was able to selectively maintain a more primitive population characterized by increased colony forming ability compared to the non-DWJM conditions. Since the 3D culture conditions include 2 compartments: the non-adherent cells in suspension, and the adherent cells embedded within DWJM, we characterized these two compartments separately, as studies have shown that the niche can transmit the signals to HSPCs through adhesion molecules binding to their ligands including ECM proteins (Kerst, Sanders et al. 1993, Coulombel, Auffray et al. 1997).

C-kit/SCF and CXCR4/SDF-1 are probably the most well-established key signaling

regulators of HSC maintenance (Sugiyama, Kohara et al. 2006, Ding, Saunders et al. 2012, Ding and Morrison 2013, Greenbaum, Hsu et al. 2013), thus we sought to find out whether there were any changes of c-kit or CXCR4 expression in the cells cultured in different conditions. We found that adhesion of $CD34^+$ cells to DWJM resulted in higher c-kit expression; we further analyzed the c-kit expression within $CD34^+$ $CD38^-$ population. The fact that adhesion to DWJM prevented the loss of c-kit within $CD34^+CD38^-$ population is one reason of the increased self-renewal.

As ECM in BM serves as a reservoir of many cytokines and growth factors, along with the fact that our previous mass-spec study found that some soluble signaling molecules such as TGF- β and protein kinase are present in DWJM even after many processing procedures including strong washing, it is likely that DWJM binds to SCF and accordingly prevents the diffusive loss or even the c-kit/SCF internalization by cells upon ligand-receptor interaction (Jahn, Seipel et al. 2002). This might improve the downstream signaling initiated by this receptor protein kinase.

CXCR4/SDF-1 axis is considered the main pathway affecting the homing efficiency of HSCs after transplantation (Lapidot, Dar et al. 2005). Since the CXCR4 expression is extremely rare in freshly isolated $CD34^+$ cells, it is perhaps one reason causing the delayed engraftment in UCB transplantation due to low homing efficiency. Interestingly, the two compartments of our DWJM showed different abilities in inducing CXCR4 expression: the $CD34^+$ cells in non-adherent compartment had significantly higher CXCR4 frequency, while no increase in CXCR4 expression in the adherent population

was found. Our transmigration study further confirmed that transmigration potential was not induced by adhesion to DWJM.

As BM is the main site for adult hematopoiesis, it is important that the engineered BM niche is capable of support the multi-lineage differentiation of HSCs. While cord blood $C34^+$ cells were able to sustain the differentiation of almost all the cell types in both myeloid and lymphoid lineages, DWJM significantly promoted megakaryocyte differentiation by at least two fold. Since some recent works indicate that these megakaryocytes play an important role in HSC quiescence by secreting TGF-β1 , CXCL4 and TPO (Bruns, Lucas et al. 2014, Nakamura-Ishizu, Takubo et al. 2014, Zhao, Perry et al. 2014), it might also be one reason that these cells derived from $CD34^+$ cells prevented the differentiation-prone proliferation of HSCs. This observation is important since delayed platelet recovery is prevalent among patient undergoing cord blood stem cell transplantation (Gluckman 2000) . Using mononuclear cells cultured in the presence of DWJM will potentially help shorten the time for platelet recovery, overcoming one of the main drawbacks of cord blood stem cell transplantation.

In conclusion, our novel DWJM based culture system provides two main components of the bone marrow niche: BM stromal cells and ECM. The *in vitro* DWJM-based system of the bone marrow niche allows us to study the influence of different niche components on normal and malignant hematopoiesis including proliferation, differentiation, and self-renewal as well as expand HSPCs for clinical use.

Chapter 3

A novel extracellular matrix-based leukemia model derived from Wharton's jelly

Abstract

Relapse of Acute Myeloid Leukemia (AML) results from the survival of chemotherapy-resistant and quiescent leukemia stem cells (LSC). These LSCs reside in the bone marrow microenvironment comprised of other cells and extracellular matrix (ECM) that facilitates LSC quiescence through expression of cell adhesion molecules. We used decellularized Wharton's jelly matrix (DWJM), the gelatinous material in the umbilical cord which contains many components of bone marrow extracellular matrix including collagen, fibronectin, lumican, and hyaluronic acid (HA), as a scaffolding material to culture leukemia cells. Leukemia cells cultured in DWJM demonstrated decreased proliferation without undergoing significant differentiation. In addition, leukemia cells showed changes in morphology with a spindle-shaped appearance after culture in DWJM. Also, there was an increase in $ALDH^+$ cell population. When treated with a high dose of doxorubicin, leukemia cells in DWJM demonstrated less apoptosis compared to cells in suspension. Serial colony forming unit (CFU) assay indicated leukemia cells in DWJM resulted in increased colony-forming ability both after primary and secondary plating. This could be explained by an induction of a cell adhesion molecule N-cadherin expression. Our data suggest that DWJM could serve as an ECM-based model to study AML stem cell-like cell behavior and chemotherapy sensitivity.

Introduction

Acute myeloid leukemia (AML) is a heterogeneous hematopoietic malignancy characterized by an aberrant clonal expansion of undifferentiated myeloid blasts. Studies have shown that leukemic stem cells (LSCs) account for relapse after chemotherapeutic treatment (Wang and Dick 2005). Like normal stem cells, LSCs maintain their self-renewal ability while generating clonogenic leukemic progenitors which finally lead to the production of leukemic cells (Hope, Jin et al. 2004). Because anti-proliferative chemotherapeutic agents commonly target the rapidly cycling leukemic cells which are mainly in the circulation, it is important to develop therapeutic strategies which eliminate the LSCs in the bone marrow where they share the “hematopoietic niche” along with normal hematopoietic stem cells (HSCs) (Lo Celso, Fleming et al. 2009). LSCs hijack the hematopoietic stem cell niche to their advantage. The LSC niche, similar to the hematopoietic niche, is a three-dimensional (3D) microenvironment composed of bone marrow stromal cells and extracellular matrix (ECM) components like collagen, fibronectin and tenascin (Lo Celso, Fleming et al. 2009). They create compartments that not only provide the structural support to the cells in the bone marrow, but also provide chemokines and cytokines which are important in regulating HSC and LSC self-renewal, trafficking, proliferation and differentiation (Lane, Scadden et al. 2009).

Currently, most leukemia in vitro studies are based on conventional two-dimensional (2D) cultures on tissue culture polystyrene (TCP) dishes/ flasks, or animal models. These models are useful in elucidating some of the molecular mechanisms of leukemia initiation and progression. However, the 2D culture system lacks the

leukemia-microenvironment interaction in the 3D bone marrow microenvironment, and the LSC frequently differentiate and lose their “stem-ness”, while the expensive *in vivo* animal models cannot fully mimic the human bone marrow microenvironment which regulates leukemia proliferation, differentiation, and chemoresistance. Thus, the development of an *in vitro* 3D model that possesses the bone marrow mechanical and molecular properties could be useful in overcoming these challenges in in vitro leukemia culture systems. Previously our lab established a 3D stromal based model for testing AML chemoresistance. Briefly, in co-culture with primary bone marrow mesenchymal stromal cells (MSCs) seeded in PGA/PLLA scaffold, AML cells showed increased chemoresistance. Our next goal is to test whether another main component of bone marrow stem cell niche, the ECM, could also play a role in chemoresistance, or even be able to enrich a subpopulation with stem cell properties (Aljitawi, Li et al. 2014). For the last few years we have been interested in studying decellularized Wharton’s jelly matrix (DWJM) for regenerative medicine applications (Aljitawi, Xiao et al. 2013). As previously described, Wharton’s jelly is the gelatinous connective tissue of the umbilical cord that mainly supports blood vessels in umbilical cord. Its components such as collagen, fibronectin, hyaluronic acid (HA), and sulphated proteoglycan (Sobolewski, Bankowski et al. 1997, Franc, Rousseau et al. 1998) also exist in the bone marrow hematopoietic niche (Lo Celso, Fleming et al. 2009). In addition, DWJM could also serve as a reservoir for some soluble growth factors such as Insulin-like growth factor 1 (IGF-1), Fibroblast Growth Factor (FGF), Transforming growth factor beta 1 (TGF-β1), Epidermal growth factor (EGF), and Platelet-derived growth factor (PDGF) (Sobolewski, Malkowski et al.

2005). Thus, we hypothesized that our DWJM can be used to establish an *in vitro* model to study LSCs. We evaluated decellularized Wharton's jelly matrix (DWJM) as a 3D extracellular matrix (ECM) model to study leukemia. Briefly, we investigated the growth pattern including proliferation, viability, morphology and myeloid differentiation of 3 human leukemia cell lines HL60 (acute promyelocytic leukemia), Kasumi-1 (acute myeloblastic leukemia), and MV411 (biphenotypic B myelomonocytic leukemia) in the model, to reflect the different subtypes of AML. We also compared the drug resistance and stem cell characteristics of leukemia cells in the model with leukemia cells in suspension. We found that AML cells grown in our ECM model using DWJM had preserved LSC-like characteristics, suggesting that DWJM may prove useful for the further characterization and targeting of LSC.

Materials and Methods

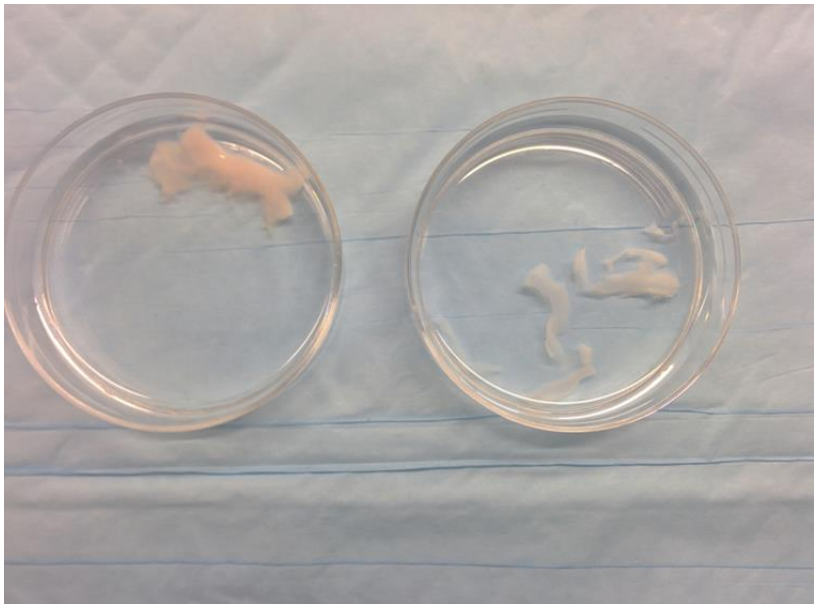
1. Cell culture

Human AML cell lines HL60, Kasumi I and MV 411 (ATCC, Manassas, VA) were maintained in T 75 tissue culture flasks with Advanced Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco) supplemented with 5% fetal bovine serum (Sigma-Aldrich) and 1% penicillin/streptomycin (pen/strep) (Life Technologies). Cells were maintained at 37°C in a fully humidified 5% CO2 incubator for the experiment.

2. DWJM scaffolds preparation

Figure 9. Characterization of DWJM. (A) DWJM scaffolds of larger chunks (left) and small wafers (right). The small wafers were used as scaffolds for leukemia cells. (B) Comparison of main components in DWJM and bone marrow ECM.

A



B

	Bone marrow	DWJM
Collagen	+	+
Fibronectin	+	+
Laminin	+	+
Chondroitin	+	
Hyaluronate	+	+
Dermatan	+	
Heparan sulfate	+	+

The preparation of DWJM was described above in Chapter 2. After decellularization, the DWJM were cut into small scaffolds (Figure 9A). This ECM material is composed of many proteins and glycosaminoglycans which are also found in bone marrow microenvironment (Figure 9B).

3. Seeding DWJM wafers with AML cell lines

Before seeding, cryopreserved DWJM wafers were thawed, washed in phosphate buffered saline (PBS) three times, and pre-incubated with Advanced RPMI overnight. AML cells ($2*10^5$ cells/well) were seeded into DWJM wafers in 24-well non-tissue culture treated plates with 60% area of each well covered by DWJM. Culture plates were then placed in an incubator at 37°C with 5% CO2 and maintained in Advanced RPMI with 5% FBS for 7 days and half the medium was changed every other day. AML cells in suspension ($2*10^5$ cells/well) cultured under the same conditions were used as control. In AlamarBlue assay, CellTrace proliferation and Ki67 immunohistology sample preparation, cells were maintained in RPMI 1640 (Sigma-Aldrich) with 10% FBS for HL60 and MV411, and 20% FBS for Kasumi I.

4. AlamarBlue assay

To assess the proliferation of AML cells, DWJM wafers with cells were transferred to new 24 well plates and washed with PBS three times 4 hours prior to proliferation assessment. Ten percent alamarBlue (Biocentric) was added into each well. After 4 hours, 100 µl supernatant of each well was aspirated to a new well of a 96 well plate and

fluorescence was measured by micro-plate reader with excitation wavelength at 530nm and emission wavelength at 590 nm.

5. CellTrace proliferation assay

To monitor the cell division of leukemia cells in both suspension and DWJM, cells were labeled with CellTrace Violet (Life Technology) before seeding. Briefly, cells were washed and resuspended with PBS at the concentration of 10^6cells/ml, and CellTrace Violet stock solution was added in a final concentration of 1µl/ml. After incubation at room temperature for 20 minutes 5ml of PBS with 10% FBS were added and incubated for 5 minutes followed by centrifugation to obtain pellets. Cells were resuspended in culture medium and cultured in either suspension or DWJM. Cell division was determined by flow cytometry soon after seeding and after 7 days of culture. To isolate cells from DWJM, we washed wafers in PBS, and then used collagenase II to digest DWJM at 37°C.

6. Cell viability

Cell survival in DWJM was measured by Vi-CELL Series Cell Viability Analyzer (Beckman Coulter) which is based on Trypan Blue dye exclusion. Cells in each DWJM wafer were released by treating with 1ml 0.002g/ml collagenase II (Worthington) for about 2 hours in 37°C. The released cells were assessed for viability according to manufacturer's recommendations.

7. Histology and Immunohistochemistry

For morphological analysis, wafers were washed with PBS three times, fixed in 4% PFA, embedded in paraffin, sectioned and stained with hematoxylin and eosin, and visualized under the microscope using Olympus BX40 microscope and pictures were obtained using DP72 digital camera.

For immunohistochemistry, formalin-fixed, paraffin-embedded sections (4μm) were subjected to immunohistochemical staining of Ki67 and N-Cadherin. Briefly, after deparaffinization and rehydration, tissue sections were treated using citrate buffer (pH 6.0) for 5 min in a pressure cooker for antigen retrieval. Hydrogen peroxide (3%) was then applied to the sections to quench endogenous peroxidase activity. Sections were then incubated with primary antibodies against Ki67 (clone MIB-1; 1:200 dilution; Dako, Carpinteria, CA, USA) and N-Cadherin (1:100 dilution; Immuno-Biological Laboratories Co., Minneapolis, MN, USA) for 30 min at room temperature respectively. After extensive rinsing, Ki67 sections were incubated with anti-mouse HRP-labeled polymer (EnVision ^{TM} + System, Dako, Carpinteria, CA, USA), while N-Cadherin sections are incubated with MACH 2^{TM} Rabbit HRP-Polymer (Biocare Medical, Concord, CA, USA). Finally, the staining was visualized by DAB (Dako, Carpinteria, CA, USA), and nuclei were counterstained with hematoxylin. All immunohistochemical staining was performed using the IntelliPATH FLX ^{TM} Automated Stainer (Biocare Medical, Concord, CA, USA) at room temperature. Slides were assessed by Automated Cellular Imaging System (ACIS).

8. Treatment with chemotherapy

After 7 days, both cells cultured in suspension and cells in DWJM were treated with 50μM of doxorubicin hydrochloride (Sigma-Aldrich) for 48 hours. For cells in suspension, culture medium was removed, and chemotherapeutic agents were added in fresh medium. For cells in DWJM, scaffolds were transferred into new 24-well plates, washed with PBS three times followed by adding chemotherapeutic agent in culture medium.

9. Apoptosis assay

Apoptosis in leukemia cells was measured by flow cytometry using Annexin V-Alexa 568 (Invitrogen, USA) and DAPI (Invitrogen, USA) staining. Prior to flow cytometry analysis, cells in DWJM wafers were released as described previously and 10^5released cells as well as cells in suspension were stained with DAPI and Annexin V according to manufacturer's recommendations, and data were acquired within 1 hour by using LSR II (BD Biosciences), and analyzed by FlowJo software.

10. Aldefluor assay

Aldehyde dehydrogenase (ALDH) activity was examined by using Aldefluor reagent (Stem Cell Technologies) according to the manufacturer's protocol followed by flow cytometry. Cells negative for propidium iodide (PI) staining were considered positive for ALDH based on a negative control using ALDH inhibitor diethylaminobenzaldehyde (DEAB). Data were analyzed within 1 hour by using LSR II (BD Biosciences).

11. Analysis of differentiation marker

Expression of CD11b was measured by flow cytometry. 10^5 cells cultured in suspension or in DWJM were harvested and incubated with FITC-conjugated anti-human CD11b (Abcam) for 10min at room temperature. After staining cells were washed and resuspended in 400µl PBS and analyzed by flow cytometry by using LSR II (BD Biosciences).

12. Colony Forming Unit (CFU) Assay

Cells were plated on 35 mm dishes (500 cells/dish for Kasumi I, and 300 cells/dish for HL60 and MV411) in triplicate in MethoCult® H4434 Classic methylcellulose (Stemcell Technologies, Vancouver, Canada). Cells were washed and resuspended in RPMI 1640 medium. After determining cell number and viability by trypan blue, cell density were adjusted at a concentration of 10 cells/µl, and cells were added into methylcellulose followed by adding 1ml cell- methylcellulose mix into each dish. After 12-14 days of incubation at 37ºC and 5% CO2, colonies consisting of >30 cells were counted, then harvested and replated in methylcellulose. After another 12-14 days, colonies were counted.

13. Data analysis

All data analyses are done with Graphpad Prism 6 (GraphPad Software, Inc.) and presented as means ± standard deviation (SEM) and significance are determined using Student's *t*-tests. Statistical significance was determined by a statistical threshold of $p < .05$.

Results

DWJM decreases leukemia cell proliferation while maintaining cell viability

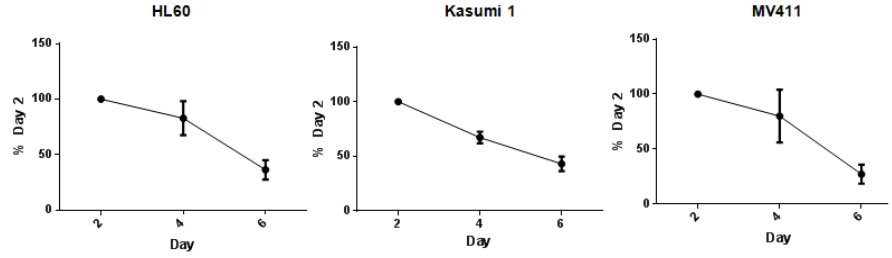
Leukemia cell proliferation in DWJM was characterized by AlamarBlue assay on days 2, 4, and 6 after seeding. All the three cell lines HL 60, Kasumi I and MV411, showed decreased proliferation while growing in DWJM during the first week after seeding (Figure 10A). Since it is possible that the decreased proliferation was due to the lower cell viability in DWJM, accordingly we also measured cell viability at the same three time points. We found that all the three cell lines maintained nearly unchanged viability at the three time points (Figure 10B). To further compare cell division in DWJM to suspension, we pre-labeled seeded leukemia cells with CellTrace Violet reagent prior to culture (Figure 10C). Consistent with AlamarBlue assay, after 7 days, cells in suspension lost more fluorescence compared to cells in DWJM, indicating that cells in DWJM turned to be more quiescent. At the same time we used Annexin V/PI staining to assess cell apoptosis. There was no significant difference between cells in suspension and DWJM (Figure 10D).

Leukemia cells in DWJM switch to spindle-shaped morphology

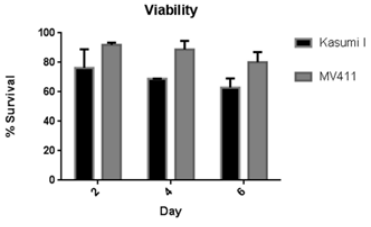
We noticed that HL 60 and Kasumi I cells cultured in DWJM tend to change their morphology, switching from round, which is the morphology of AML cells in suspension, to spindle shape 7 days after seeding (Figure 11A and B). In general, there were more spindle-shaped cells than round cells per high power field (HPF) (Figure 11C). The round

Figure 10. DWJM decreases leukemia cell proliferation while maintaining cell viability. (A) HL60, Kasumi 1 and MV411 cells were seeded and allowed to adhere and penetrate DWJM for 2 days. Then DWJM with cells were taken to a new plate, and AlamarBlue assay were used to assess cell proliferation on day 2, 4, and 6. Data is normalized to day 2 fluorescence reading. (B) Collagenase II was used to release cells embedded in DWJM, and cell viability was assessed by Vi-CELL based on trypan blue exclusion on day 2, 4, and 6. All values represent means ± SEM. (C) Violet intensity of HL60 and MV411 in DWJM and suspension before and 7 days after seeding. D: Apoptosis and necrosis of HL60 and MV411 cultured in suspension or DWJM by co-staining of AnnexinV/PI and flow cytometry analysis. Results are shown as density plots (upper) and quantification (bottom). Data represent means ±SEM. Experiment done in triplicate.

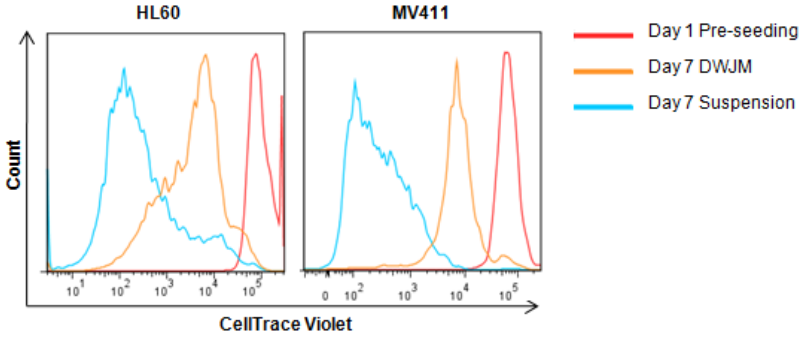
A


HL60
Kasumi 1
MV411
% Day 2
Day

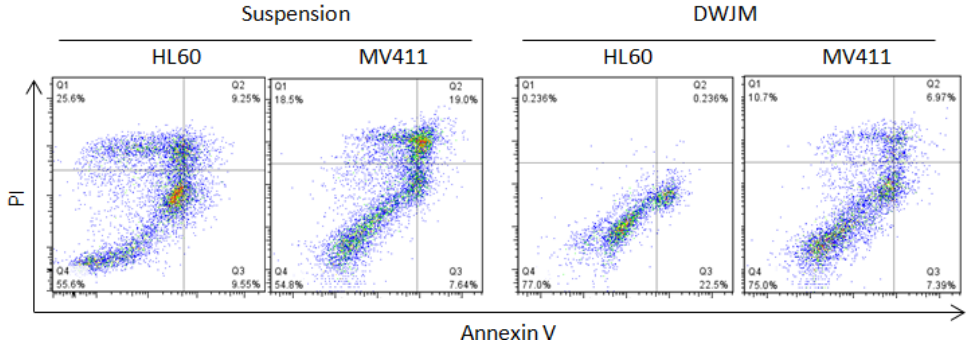
B

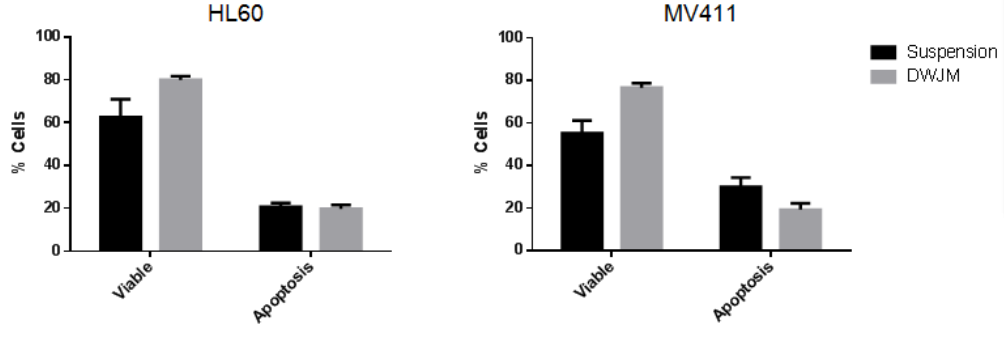

Viability
% Survival
Day
Kasumi I
MV411

C


HL60
MV411
Count
CellTrace Violet
Day 1 Pre-seeding
Day 7 DWJM
Day 7 Suspension

D


Suspension
DWJM
HL60
MV411
HL60
MV411
PI
Annexin V


HL60
MV411
% Cells
Viable
Apoptosis
Suspension
DWJM

cells are mostly seen in the open spaces within the matrix, while the spindle shaped cells are mostly embedded inside the matrix.

DWJM keeps leukemia cells undifferentiated while increasing the stem/progenitor cell marker ALDH+ population

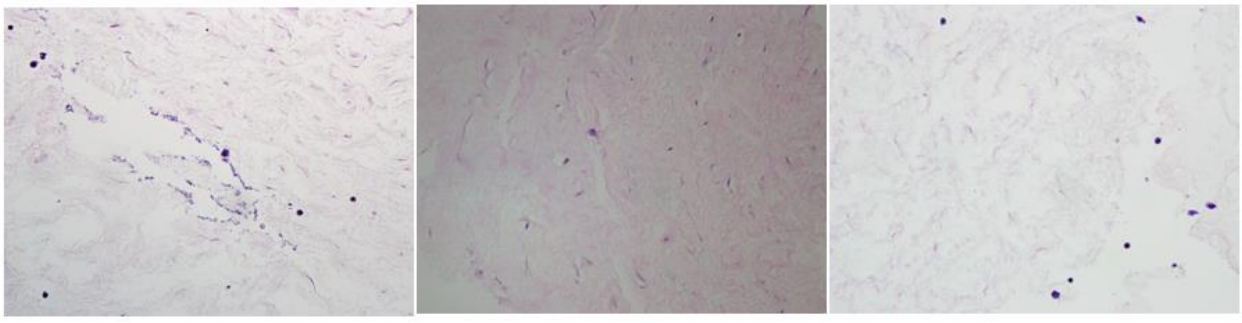
As CD11b is a common myeloid marker (Dziennis, Van Etten et al. 1995), we evaluated CD11b expression to estimate differentiation levels of the three AML cell lines. Neither the cells in suspension nor cells in DWJM expressed CD11b, indicating that the matrix maintains leukemia cells in an undifferentiated stage (Figure 12A). Next we examined the expression of stem cell marker ALDH. High ALDH expression has been reported in several lineages of cancer precursor cells including hematopoietic, mammary, endothelial, mesenchymal, and neural, indicating that the $ALDH^+$ population may be more tumorigenic. We found that in both Kasumi I (~7 fold, $p<0.05$) and MV411 (~2 fold, $p<0.05$), cells cultured in DWJM significantly increase ALDH activity, compared with cells in suspension (Fifure 12B), while HL60 did not show any difference (data not shown).

DWJM increases leukemia cell clonogenic ability

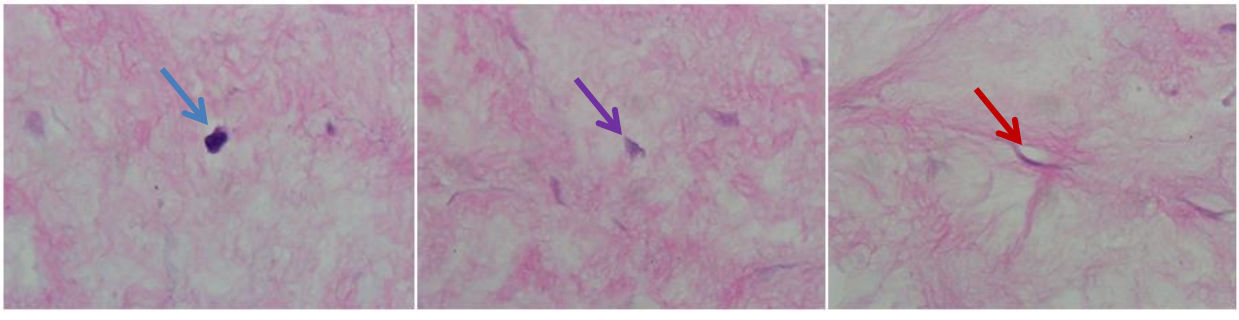
Since ALDH enriches both cancer stem and progenitor cells, to examine the self-renewal of leukemia cells cultured in DWJM, we used serial CFU assay, as primary CFU assays demonstrate progenitor potential of leukemia cells, and secondary replating correlates with self-renewal ability. In general, in all the three cell lines, colony number from both primary and secondary plating increased significantly (Figure 13). In two cases, the

Figure 11. Leukemia cells in DWJM switch to spindle-shaped morphology. (A) A representative of H&E staining of HL60 (left), Kasumi 1 (middle) and MV411 (right) cells cultured in DWJM for one week (40x). (B) Kasumi 1 cells switch from round to spindle-shaped. Left: round; middle: round-spindle; right: Spindle (100x). C: Number of different shaped cells in each high power field (HPF). Data represent means ± SEM.

A



B



C

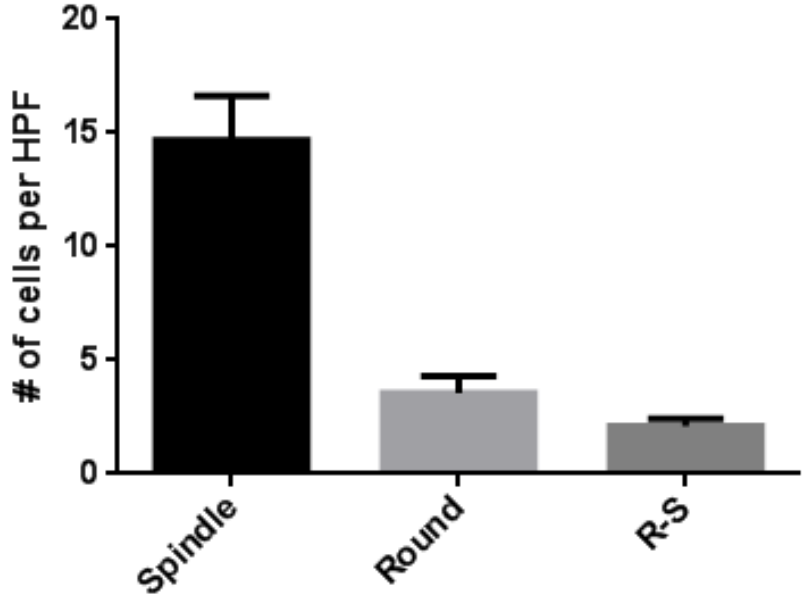
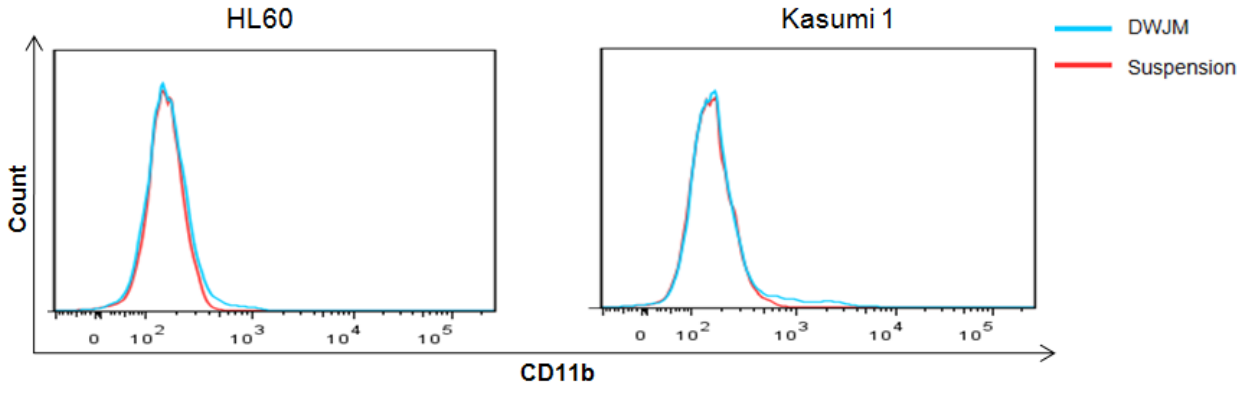
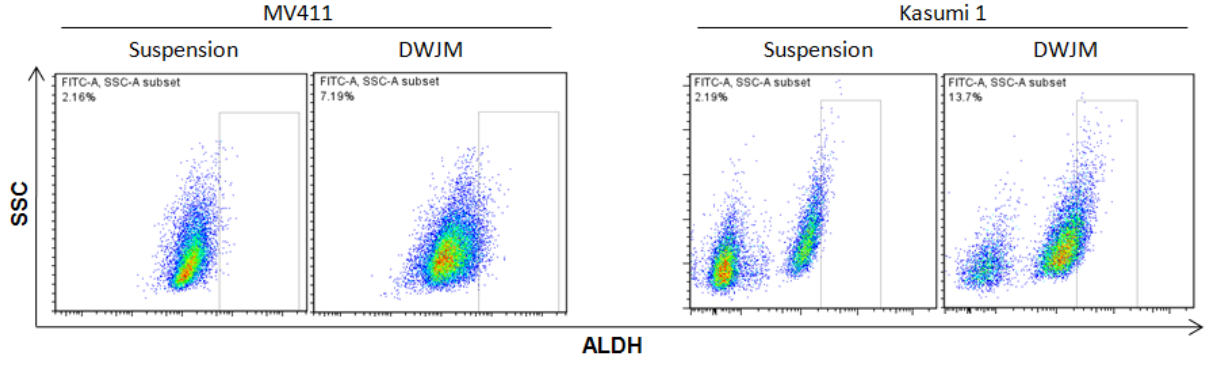


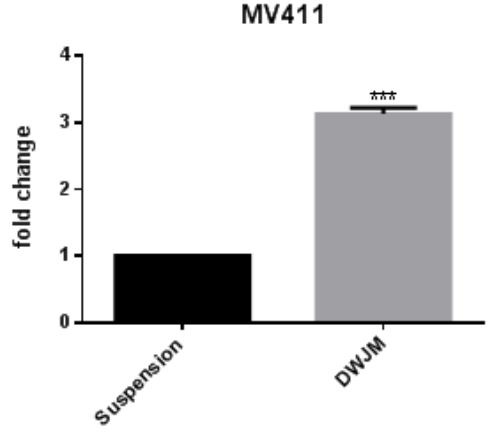
Figure 12. DWJM keeps leukemia cells undifferentiated while increasing the stem/progenitor cell marker ALDH+ population. (A) CD11b expression in HL60 and Kasumi 1 cultured in suspension and DWJM. (B) Representative density plots (upper) and quantification (bottom) of Aldefluor assay of MV411 and Kasumi 1 after 7 days cultured in suspension or DWJM. Data represent means ±SEM. Experiment done in at least duplicate.

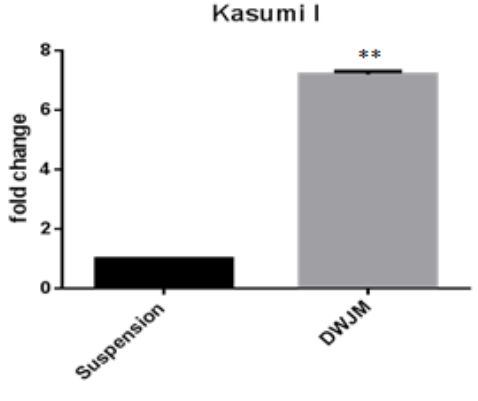
A


HL60
Kasumi 1
DWJM
Suspension
Count
CD11b

B


MV411
Kasumi 1
Suspension
DWJM
FITC-A, SSC-A subset
2.16%
7.19%
2.19%
13.7%
SSC
ALDH


MV411
fold change
***
Suspension
DWJM

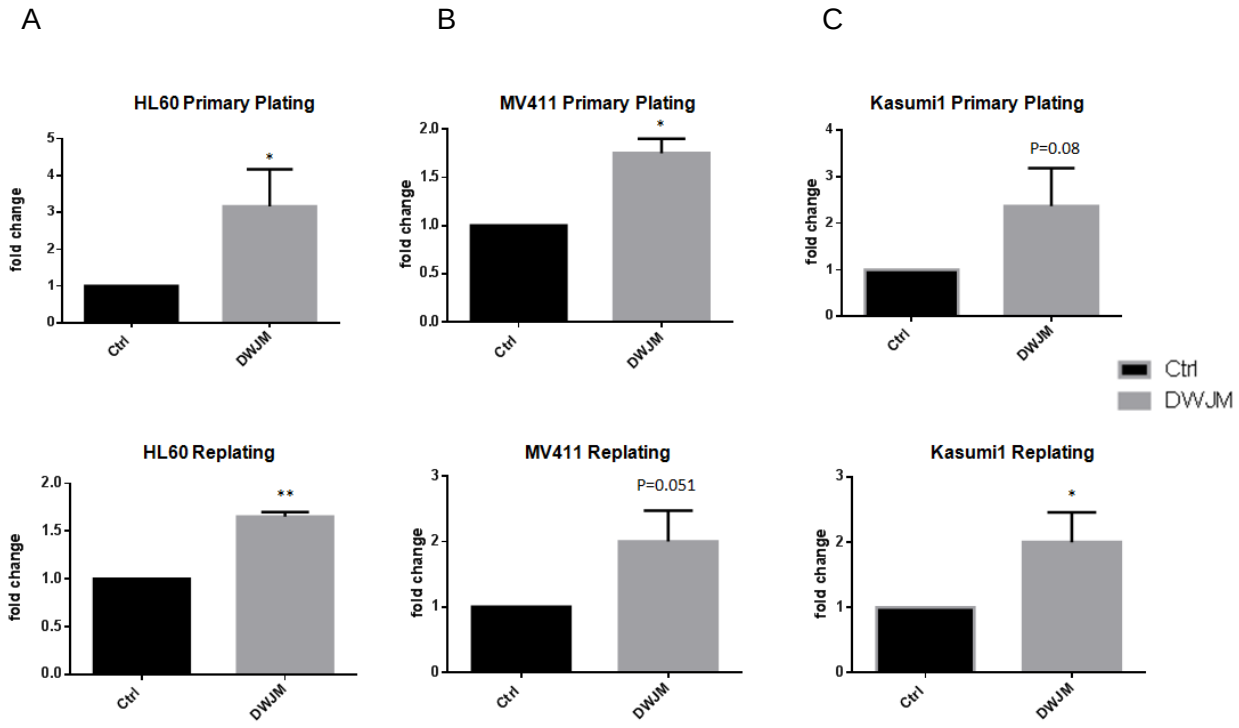

Kasumi I
fold change
**
Suspension
DWJM

increase approached statistically significant in Kasumi 1 after primary plating (figure 13C, p=0.08) and MV411 after secondary plating (figure 13B, p=0.051). In all leukemia cells cultured in DWJM they gained long-term self-renewal ability as the colony-forming ability was maintained even 28 days after their release from DWJM.

DWJM enriches leukemia cells with increased drug resistance

As leukemia stem cells are more chemoresistant, and are responsible for disease relapse after chemotherapy, we next investigated doxorubicin's effects on leukemia cells in both suspension and DWJM. We used a very high dose, 50μM, which killed more than 95% of cells in suspension after 48 hour treatment (Figure 14 A and B), to treat leukemia cells in both culture conditions. After 48h treatment, we used Annexin V/DAPI to assess cell apoptosis/necrosis. As expected, doxorubicin induced cell death mainly through apoptosis. We observed that the survival rate of cells cultured in DWJM significantly increased in all three cell lines HL60 (~2.5 fold, $p<0.5$), Kasumi I (~8 fold, $p<0.05$), and MV411 (~5 fold, $p<0.1$) (Figure 14 A and B), suggesting that culture in DWJM could have protected leukemia cells from the cytotoxicity of anti-cancer drugs. We further analyzed doxorubicin uptake by measuring its fluorescent intensity and found that the accumulation of doxorubicin decreased in leukemia cells cultured in DWJM (Figure 14 C). Since N-cadherin was reported as a stem cell marker and lead to CML stem cell drug resistance by activation of Wnt signaling, we further analyzed N-cadherin expression and found that cells in DWJM increased N-cadherin expression compared to cells in suspension (Figure 14D).

Figure 13. DWJM increases leukemia cell clonogenic ability. Clonogenic ability of HL60 (A), MV411 (B), and Kasumi 1 (C) after cultured in DWJM or suspension for 7 days. Data represent means ±SEM. Experiments done in triplicate. $^{*}P<0.05$; $^{**}P<0.01$.

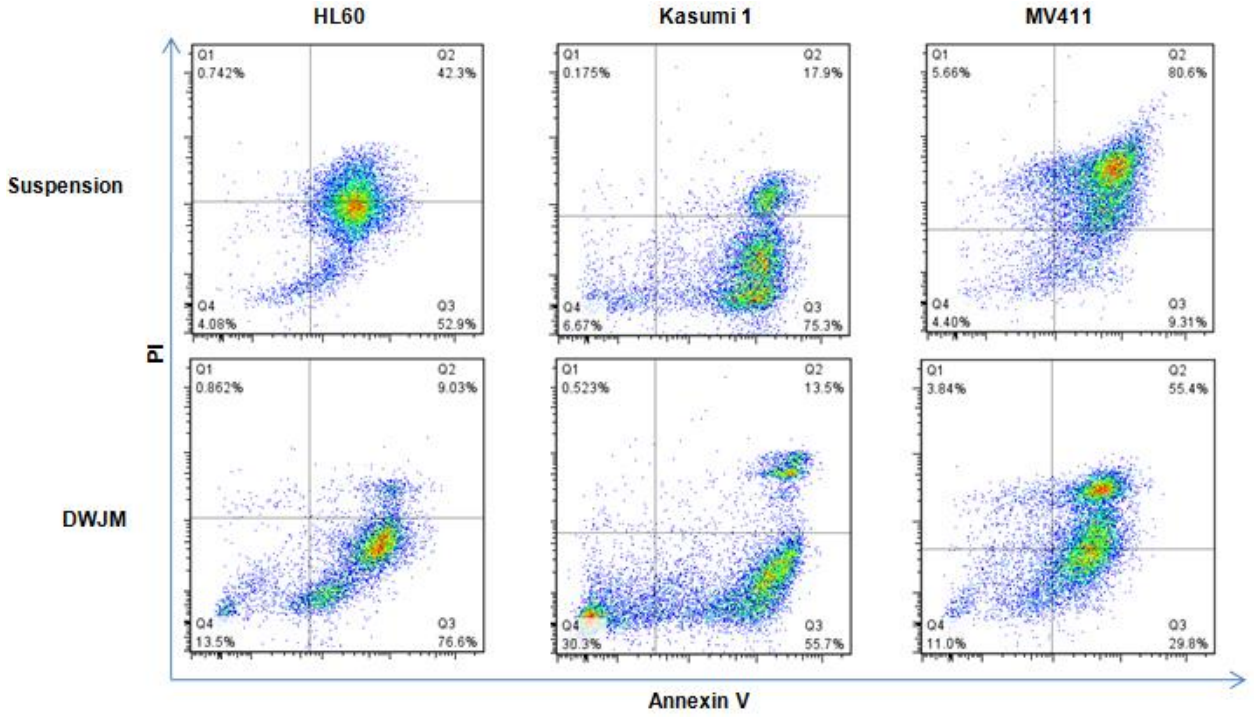

A
B
C
HL60 Primary Plating
MV411 Primary Plating
Kasumi1 Primary Plating
fold change
Ctrl
DWJM
*
P=0.08
HL60 Replating
MV411 Replating
Kasumi1 Replating
**
P=0.051

Discussion

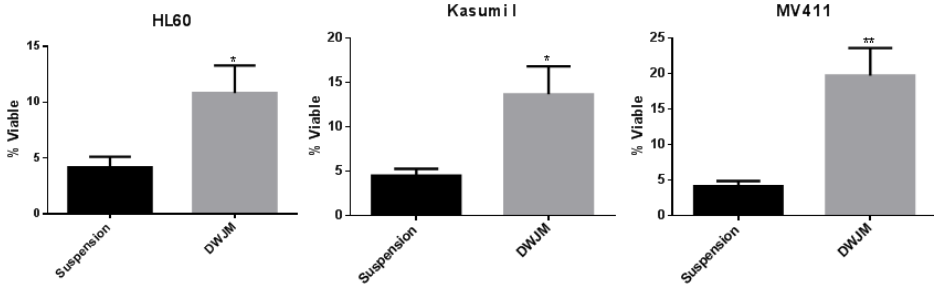
In this study, we used DWJM as an ECM model to develop a novel 3D in vitro leukemia culture system representing different AML cell lines. Our first finding is that DWJM supports leukemia cell migration and maintains cell viability. The comparatively lower cell division rate, along with the maintenance of cell viability, suggests that this matrix keeps cells in a more quiescent state. Since the quiescent subpopulation of AML cells counted for the disease relapse after chemotherapy, our findings suggest that DWJM might be able to enrich leukemia cells with stem cell characteristics. We next measured the expression of CD11b, a myeloid differentiation marker to see whether DWJM could maintain leukemia cells in an undifferentiated state. We found the expression of CD11b was negligible both in suspension and DWJM, indicating that such ECM material does not cause differentiation of leukemia cells. We also examined intracellular ALDH activity since it has been reported to enrich normal stem cells including hematopoietic stem cells (Kastan, Schlaffer et al. 1990, Storms, Trujillo et al. 1999), cancer stem cells in breast cancer cell lines (Croker, Goodale et al. 2009), as well as leukemia stem cells in patient bone marrow samples (Gerber, Smith et al. 2012). We found that after culturing leukemia cells in DWJM, there was an increase in $ALDH^+$ population in both Kasumi 1 and MV411. These findings suggest that our system favored LSC-like phenotype and behavior over leukemia cell differentiation and proliferation.

Figure 14. DWJM enriches leukemia cells with increased drug resistance. (A) Representative density plots and (B) quantification of apoptosis and necrosis in HL-60, Kasumi I and MV411 in either DWJM or suspension were measured after treated with 50μM doxorubicin for 48 hours. Data represent means ± SEM. Experiment done in triplicate. *P<0.05; **P<0.01. (C) Doxorubicin uptake in HL60 (left) and MV411 (right) cells after treatment. (D) N-cadherin expression in HL60 (left) and MV411 (right) after 7 days culture in suspension (blue) and DWJM (red).

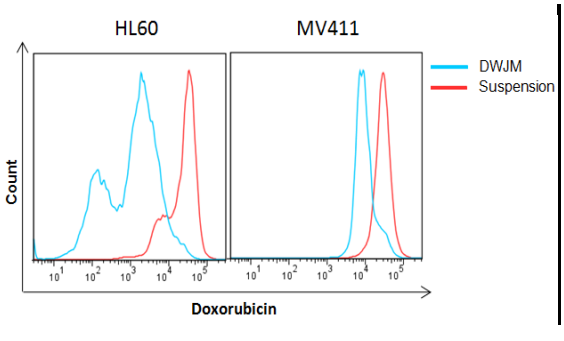
A


HL60
Kasumi 1
MV411
Suspension
DWJM
PI
Annexin V
Q1 0.742%
Q2 42.3%
Q4 4.08%
Q3 52.9%
Q1 0.175%
Q2 17.9%
Q4 6.67%
Q3 75.3%
Q1 5.66%
Q2 80.6%
Q4 4.40%
Q3 9.31%
Q1 0.862%
Q2 9.03%
Q4 13.5%
Q3 76.6%
Q1 0.523%
Q2 13.5%
Q4 30.3%
Q3 55.7%
Q1 3.84%
Q2 55.4%
Q4 11.0%
Q3 29.8%

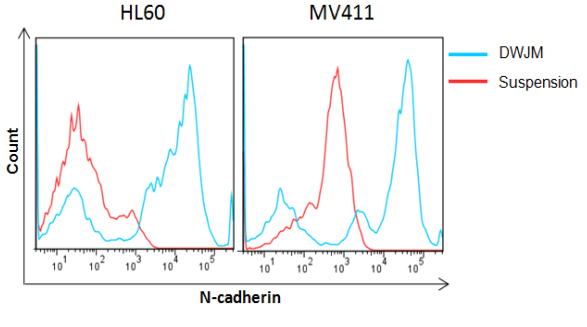
B


HL60
Kasumi l
MV411
% Viable
Suspension
DWJM

C


HL60
MV411
DWJM
Suspension
Count
Doxorubicin

D


HL60
MV411
DWJM
Suspension
Count
N-cadherin

Like normal HSCs, leukemia stem cells also possess strong self-renewal ability and it is required for the long-term disease maintenance. To assess the self-renewal ability of leukemia cells in DWJM, we used serial CFU assay by counting colony numbers formed after both primary plating and secondary replating, since it has been reported as an ideal *in vitro* assay complementary to serial transplantation (Lin, Wang et al. 2010). Our result showed that the clonogenic ability of leukemia cells was increased by DWJM in both primary and secondary plating, meaning the self-renewal was long-lasting.

Chemoresistance is one of the major characteristics of cancer stem cells, and is also a main obstacle leading to the failure of chemotherapy (Holohan, Van Schaeybroeck et al. 2013). It is well-known that some components in the microenvironment play an important role in inducing cancer cell drug resistance (Konopleva and Jordan 2011). Previous studies also found that different types of cancer cells increased drug resistance when cultured in 3D collagen gels (Dangi-Garimella, Sahai et al. 2013, Leung, Moraes et al. 2015), and lymphoma cell adhesion to fibronectin acquired resistance to mitoxantrone (Hazlehurst, Argilagos et al. 2006). Since these components are all present in DWJM, we assessed whether DWJM could help shelter leukemia cell from chemotherapy. (Asou, Tashiro et al. 1991, Gu, Cimino et al. 1992, Jackson, Schenker et al. 1994, Tsuruo, Naito et al. 2003, Lee, Kuo et al. 2005, Palle, Frost et al. 2005, Cordo Russo, Garcia et al. 2008, Brown, Reinke et al. 2011, Louderbough and Schroeder 2011, Lompardia, Papademetrio et al. 2013). The fact that more cells cultured in DWJM, compared to suspension, survived after high dose of doxorubicin treatment confirmed our hypothesis,

and this was at least partly because of the decreased intake of this chemotherapeutic reagent.

We also saw a morphology change while cells were growing in DWJM—there were more spindle-shaped cells rather than their natural, round shape in cell suspension and circulation in human body. Similarly, Mochmann et al. found that the spindle-shaped leukemia cells were more chemoresistent (Mochmann, Neumann et al. 2013). This is consistent with our findings that this model enriched for spindle cells and cells cultured in DWJM were more chemotherapy resistant. In addition, we observed that leukemia cells, in DWJM strongly express N-cadherin, a chemoresistance mediator, while we could hardly see N-cadherin in cells cultured in suspension.

In conclusion, our ECM model using DWJM supports leukemia cell growth and enriches a stem-like population. DWJM should be further examined as a platform for leukemia stem cell research.

Chapter 4

Summary

Hematopoietic stem cells (HSCs) are widely used in cell and gene therapies to treat hematologic and non-hematologic diseases mainly by transplanting HSCs to the affected host. However, the limited number of such cells, especially in the case of umbilical cord blood transplants, is a barrier for using HSCs in clinical applications. Therefore, *ex vivo* expansion of HSCs, especially umbilical cord blood HSCs, overcomes this barrier and provides an adequate number of HSCs to treat patients.

As bone marrow (BM) is the main organ for adult hematopoiesis as well as the location of most HSCs, engineering a BM hematopoiesis niche *in vitro* provides a simplified but useful tool to study the biology of HSCs and provides a model to expand HSCs for potential clinical use. The main purpose of my dissertation was to use a novel natural extracellular matrix (ECM) biomaterial, decellularized Wharton's jelly matrix (DWJM), to engineer a BM niche and characterize umbilical cord blood HSCs (CD34+ cells) behavior in the DWJM-based culture system. Further, I examined the effects of other BM niche components; BM stromal cells (MSCs) and soluble cytokines and growth factors, on cell proliferation, viability, clonogenic ability, stem cell phenotype, homing potential, and multi-lineage differentiation. Since leukemia stem cells (LSCs) hijack the BM niche and its' components, we also cultured three leukemia cell lines representing different leukemia subtypes, in DWJM, to further elucidate the influence of DWJM on the maintenance of leukemia cell stem-ness.

In our HSC study, we chose to use a commercially available cytokine cocktail cc110 consisting of Flt3L, SCF, and TPO in all culture conditions as HSCs did not survive in serum-free culture medium(data not shown). Firstly, we have proved that DWJM is a

biomaterial suitable for engineering BM hematopoietic niche since HSCs could be maintained viable during 7-day culture. Similarly the leukemia cells did not show a change in viability when cultured in DWJM. We next analyzed stem/progenitor self-renewal by colony-forming unit assay, HSC phenotype by flow cytometry, and homing potential by investigating CXCR4/SDF-1 axis by both flow cytometry and transwell assay. Our results indicate that umbilical cord blood CD34+ cells cultured in the presence of DWJM demonstrate stem-like behavior evident by their quiescence while maintaining their clonogenic ability, increased C-kit^{+} population within the CD34^{+}CD38^{-} HSPCs, and stronger homing potential related to increased CXCR4 expression and improved migration toward SDF-1. The role of DWJM in supporting stem-ness was further demonstrated by the leukemia cell behavior when leukemia cells were cultured in this ECM material. Like in the case of HSCs, DWJM enriched a sub-population of leukemia cells with stem cell-like characteristics including an increased quiescent population, an enhanced ALDH expression in the cultured cells, enhanced clonogenic ability, and increased drug resistance. Drug-resistance in our model was associated decreased intracellular drug availability after treatment. Also, cultured cells preferentially exhibited spindle-shaped phenotype. The induction of N-cadherin in the cultured cells is a potential mechanism for stem cell phenotype. The downstream signaling pathways of N-cadherin including PI3K signaling and Wnt pathway is worth study in the future. Since it has been reported that the activation of β-catenin is important in the survival of LSCs in chronic myeloid leukemia (CML), next we sought to find out whether it also plays a role in protecting LSCs in AML. By testing this, we will try to block N-cadherin in our 3D

leukemia model, and assess whether they are still drug-resistant. We can also try to over-express or knock down N-cad in the leukemia cell lines, and assess the effects of N-cadherin in the cells in cell proliferation, self-renewal, and morphology change. To study the interaction between N-cadherin and Wnt signaling, we will also analyze the expression of N-cadherin, β-catenin, and P-GSK3β, as well as the interaction of N-cadherin and β-catenin. Last but not the least, we will try to use gene expression profile to compare the change of genes in stem cell self-renewal, quiescence, cytokine signaling, adhesion, metabolism, and cell cycle in cells cultured A) in DWJM vs. suspension, and cells B) enriched in n-cadherin and (C) β-catenin.

We also found that DWJM enhances CD41+ megakaryocytic differentiation of umbilical cord blood HSCs. Our model not only provides a model to study megakaryocyte differentiation, but also helps to elucidate the role of megakaryocyte in HSC maintenance. This finding has a strong clinical relevance; by using the DWJM-based model to expand CD34+ cells for transplantation, we enhance hematopoietic reconstitution ability by alleviating the risk of delayed platelet recovery.

We also evaluated BM-MSCs role in maintaining HSC stemmness in our *in vitro* culture system. Our results indicate that BM-MSCs induce HSC differentiation and lead to impairing SFD-1 driven transmigration. Since the stromal cells are heterogeneous, further studies are need to understand the specific role of each sub-population within these cells on HSC biology and function.

In conclusion, we propose that DWJM-based model maintains HSCs while the presence of stromal cells lead to their differentiation. Thus, DWJM as an ECM biomaterial-based model can be used as a BM engineered *in vitro niche* that can be used to expand cord blood HSCs for clinical transplantation and could be utilized to study LSCs as well as leukemia drug resistance.

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